2012

BREEDING INDUCED ENDOMETRITIS IN THE MARE: THE LOCAL INNATE IMMUNE RESPONSE

ELIZABETH MORAN WOODWARD
University of Kentucky, elizabeth.woodward@uky.edu

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained and attached hereto needed written permission statements(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine).

I hereby grant to The University of Kentucky and its agents the non-exclusive license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless a preapproved embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's dissertation including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

ELIZABETH MORAN WOODWARD, Student

Dr. Mats H. Troedsson, Major Professor

Dr. Mats H. Troedsson, Director of Graduate Studies

BREEDING INDUCED ENDOMETRITIS IN THE MARE: THE LOCAL INNATE IMMUNE RESPONSE

DISSERTATION

A DISSERSTATION SUBMITTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF KENTUCKY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

ELIZABETH MORAN WOODWARD

Director: Dr. Mats H. Troedsson

ABSTRACT OF DISSERATION

Uterine inflammation after breeding is considered necessary for the clearance of excess semen and debris from the uterus. A subpopulation of mares fails to clear the inflammation in a timely fashion, and develops a persistent breeding induced endometritis (PBIE). Experiments were preformed to evaluate correlations of PBIE to endometrial quality and age. Mares of advanced age and poor endometrial quality had a higher incidence of PBIE. In addition, mares fluctuated in susceptibility to PBIE from one season to the next. The uterine inflammatory gene expression in susceptible and resistant mares within the first 24 hours after breeding was investigated. The peak endometrial cytokine gene expression occurred 6 hours after insemination, and susceptible mares were found to have a reduced response of the inflammatory modulating cytokines during this time. Intrauterine accumulation of the inflammatory byproduct nitric oxide (NO) was investigated in resistant and susceptible mares within the first 24 hours after breeding. Susceptible mares had an increase in NO accumulation over time, whereas NO accumulation in resistant mares remained relatively constant. The effects of immunomodulators on uterine inflammatory response and nitric oxide accumulation in susceptible mares was investigated. Immunomodulators decreased expression of the pro-inflammatory cytokine interleukin-1β and nitric oxide accumulation. In conclusion, endometrial quality and age are indicators of susceptibility to PBIE, and susceptibly can change from year to year. Six hours after breeding is a critical time for the development of PBIE, and susceptible and resistant mares have differential endometrial inflammatory gene expression, with susceptible mares appearing to have a defect in the inflammatory modulating immune response. Finally, treatment with immune modulators alters the IL1β gene expression and intrauterine nitric oxide accumulation, which may help to explain how they act to reduce inflammation during PBIE.

Keywords: Equine, Endometritis, Uterus, Cytokine, Endometrium

Elizabeth M. Woodward
Student's Name

July 10, 2012
Date

BREEDING INDUCED ENDOMETRITIS IN THE MARE: THE LOCAL INNATE IMMUNE RESPONSE

BY

ELIZABETH MORAN WOODWARD

Mats H. Troedsson
Director of Dissertation

Daniel K. Howe
Director of Graduate Studies

July 10, 2012
Date

“There is something about the outside of a horse that is good for the inside of a man.”

– W. Churchill

This dissertation is dedicated to the horse. From you, I have learned understanding, empathy, and compassion. Your role with the human race is often a thankless one, and you have willingly served us in so many ways. I hope that I can serve you in the ways that you have served me.

ACKNOWLEDGEMENTS

There are so many people who have contributed to my growth both professionally and personally over the last five years, and to whom I owe a big thank you. This dissertation belongs to all of you, for without you, I would not have succeeded in writing it. I would like to thank all of the people at the Gluck Center. There is so much talent in this building, and it is exciting just to be immersed in it. I would especially like to express my gratitude towards the following people:

To the graduate students: most of us have left our lives to embark on this endeavor of earning advanced degrees, leaving everyone we know and love behind us. As new (naïve) students, we are often unprepared for the types of challenges that arise during our graduate education, and can be left feeling alone and lost in the face of difficulties. We have become a family away from family, helping one another through the challenges (no matter how trivial or serious). Each of you has impacted me in some way to help me grow. For this, I thank you. There are too many of you to name, but do know that I am sincere when I say that I am grateful to have met all of you and to have developed some lasting friendships along the way. I would like to thank Dr. Juliana Campos for being such a wonderful roommate and friend, and for her encouragement to stand up for and to believe in myself.

I would like to thank all of the staff at the Gluck Center for helping me with the organizational and planning parts of my program. I would like to acknowledge Mr. Roy Leach as an invaluable asset to the Gluck Center. Roy, one of the reasons you are so successful is due to your mastering the ability to get your point across using the utmost respect and composure, while simultaneously subtly commanding respect from those

around you. I really need to learn that skill from you. To the staff at the Maine Chance Farm: thank you for your tireless efforts to take care of the horses and to help me with the mares. I have enjoyed the many hours with you, and I know how much work it is to manage so many horses and people.

To the David Horohov lab: thank you for taking me into your group as one of your own to learn some of the most important and earliest lessons that helped me to develop my ability to think like a scientist. A thank you to the Jamie MacLeod lab for socially adopting me! With you, I have developed strong friendships that I hope to maintain throughout my life. I have always felt accepted into your lab even though I don't know much about muscles or bones. I would like to extend my gratitude to the Reproduction Group: I am so excited that we have grown into such a sizable and cohesive unit. Each of you is an asset to the program and offers something to the table, and as I look around the room during our lab meetings, I can see the next generation of leaders in equine reproduction. A big thanks to Dr. Kirsten Scoggin for her patience and for keeping all of us organized, especially as we transition into a larger group. I would also like to thank Dr. Mette Christoffersen for her contributions to my projects and educational experience. I have accomplished so much more because of our collaborations.

I would like to thank my advisor, Dr. Mats Troedsson: I cannot express how happy I am to be a part of your lab. Thank you for believing in me when so many times I did not believe in myself. Your sincere concern for me not only as a successful professional but also a successful person is always evident, and with your support, I never felt that there was an obstacle to big to climb. Thanks for granting me the freedom not

only to make mistakes, but also more importantly for the freedom to learn from them. I never felt the uncomfortable (and sometimes counterproductive) pressure to be perfect, and it allowed me space to grow more than I expected.

To Dr. David Horohov: thanks for your guidance and support from the start, and for spending so many hours problem solving with me. Your dedication to science and your students is something to be admired, and I hope that I can pass that on to my own students someday. I thank you for the numerous bouts of gut-busting laughter that we have shared. Lastly, thanks for being a native Philadelphian. There is a part of you that is oh-so-Philly and it reminds me of home when I miss it. To Dr. Edward Squires: thank you for serving on my committee and offering a valuable perspective for my project. Thanks for treating me as respected member of the group and for challenging me to think outside of the box. Dr. Nancy Cox, thank you for being the strong female force on my committee. I wanted you on my committee because of my admiration towards you and your professional success. Your poise, sense of humor, and energy is contagious.

I am grateful to my cat. Oscar: I still remember the day I brought your tiny flea infested body home from MacDonald's 13 years ago. I know neither of us planned for you to leave your perfect life of freedom outside and hunting for a cramped apartment in Lexington, but I am so happy that it turned out this way. It is always the best ending to a day to come home to your greeting me at the door. Thank you for your companionship when I felt alone, and for your never-ending efforts to get me to relax and remember to enjoy life.

I would like to thank my research mares. Obviously without them, none of this would have been possible. Thank you girls, for your tolerance of my clumsy hands and

for not telling anyone about my steep learning curve! Your jobs are vital and as our teachers, you contribute to the progression of equine veterinary medicine. Most importantly, my parents and I would like to thank you for keeping me safe. I don't know who the first person was to think that transrectal examination of the reproductive tract was a good idea, but whoever it was, was not only clever, but also crazy. It is a compromising and potentially dangerous situation, and I will forever be indebted to you for keeping me in one piece during my time here.

Last but not least, I would like to extend a thank you to my amazing family: I love you. I am so fortunate to have such an incredible group of kind, gracious, genuine, intelligent and importantly, humble group of people in my life. I am a better person in your presence. To my parents: thank you for your never-ending support through this journey, and for understanding and accepting my undertakings, no matter how nonsensical they may seem to be. Gargar: thanks to you I am as tough as nails, and there is no way I would have gotten through this without that part of my character.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

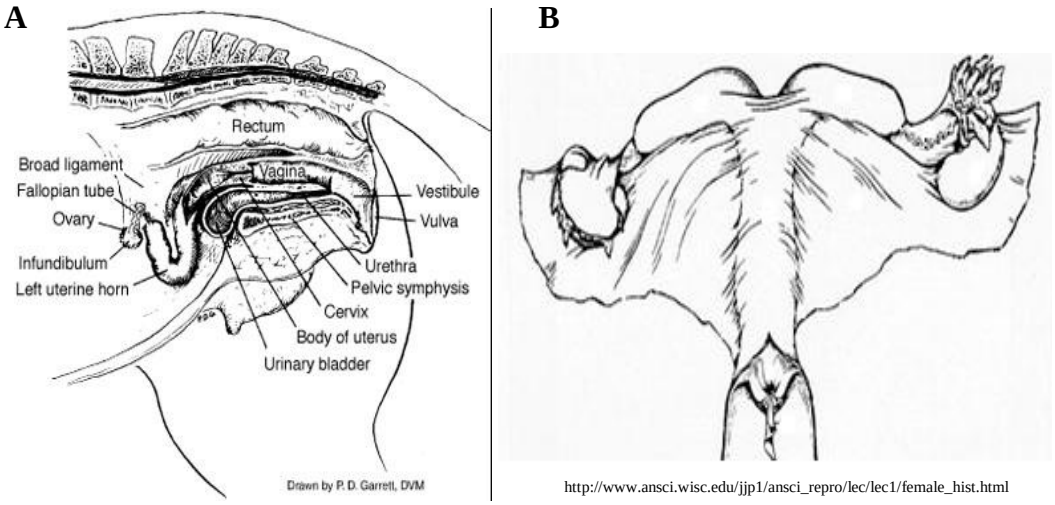
CHAPTER 1

Review of the Literature

1.1 The Reproductive Anatomy of the Mare

The equine uterus has a bicornate structure with two uterine horns connected to a shared uterine body. It is suspended in the body by the broad ligament and receives blood from the uterine and ovarian arteries, and in contrast to the upright position of the primate reproductive tract, the equine tract lays horizontally in the body. There are three major physical barriers protecting the uterus from the external environment. The outermost barrier is the vulva, and in a normal mare, the vulvar lips close completely. The transverse fold lies between the vestibule and the vagina proper, and functions as a second barrier, keeping urine from entering the upper reproductive tract. The innermost barrier protecting the uterus is the cervix, separating the vagina and the uterus (McKinnon & Voss 1993) (Figure 1.1). Reproductive hormones mediate the functionality of the cervix. During diestrus and pregnancy, progesterone causes the cervix to close tightly and produce mucous to prevent anything from entering the uterus. During estrus, estrogen and the lack of progesterone cause the cervix to soften and open, to allow for copulation and the entry of sperm into the uterus (McKinnon & Voss 1993). Prostaglandin E2 is involved in cervical ripening prior to parturition, and can be administered exogenously to soften the cervix in the cycling mare (Rigby *et al.* 1998).

Figure 1.1: The reproductive tract of the mare from the sagittal view (A) and dorsal view (B).



Like many tubular organs, the uterus is comprised of several layers. The outermost layer, (the perimetrium), aids in the suspension of the uterus within the body. The myometrium lies interior to the perimetrium and consists of two layers; an inner, circular layer and an outer, longitudinal layer. Together, these layers contract to move the contents within the uterus, whether it be spermatozoa towards the oviduct after breeding, the embryo during the migratory stages of early pregnancy, or the fetus during parturition (Ginther 1992).

The endometrium is the innermost layer of the uterus, consisting of the *lamina propria* lined by a single cell epithelial layer. The most superficial layer of the *lamina propria* is the *stratum compactum*, which contains densely packed stromal cells, while interconnecting cells in the second layer, the *stratum spongiosum*, are much more loosely arranged (Ginther 1992). Uterine glands and lymphatics are disbursed throughout the *lamina propria*. During pregnancy, the exchange of nutrients with the placenta occurs in

the endometrium. The endometrium is also involved with the uterine response to breeding, as cell signaling occurs in endometrial cells during inflammation.

Endometrial quality has become an accepted marker of uterine health (Ludwig *et al.* 2001, Schlafer 2007), and poor endometrial quality has been associated with age (Carnevale & Ginther 1992) and decreased fertility (Waelchli 1990). Healthy endometrial tissue has active, evenly distributed uterine glands, with little to no inflammatory cells. In contrast, dilated glands surrounded by layers of fibrotic cells are inactive, and are considered to impair uterine function in the degenerative uterus. Enlarged lymphatics and inflammatory cells are also commonly observed in the endometrium of mares with degenerative changes. A scale was developed to evaluate endometrial health based on the quality of glands and lymphatics, fibrotic changes, and infiltration of inflammatory cells (Kenney & Doig 1986). An endometrial grade of I is considered to be normal and healthy, with active uterine glands of normal size and even distribution throughout the stroma (Figure 1.2). Fibrosis and inflammatory cells are rare. A grade IIA endometrium may have a slight to moderate infiltration of inflammatory cells, or moderate fibrosis of the glands. Enlarged glands and lymphatic lacunae can also be observed. Although the presence of one or some of these criteria qualify an endometrium as IIA, the presence of all of the criteria changes the score to IIB, with the severity of some (but not all) of these factors as moderate to severe. If the mare has enlarged, unevenly distributed glands, and severe glandular fibrosis, lymphatic lacunae and infiltration of inflammatory cells, then her endometrium is scored as a grade III (Figure 1.3) (McKinnon and Voss 1993).

Figure 1.2: Endometrial biopsy from a grade I mare (×400 magnification).

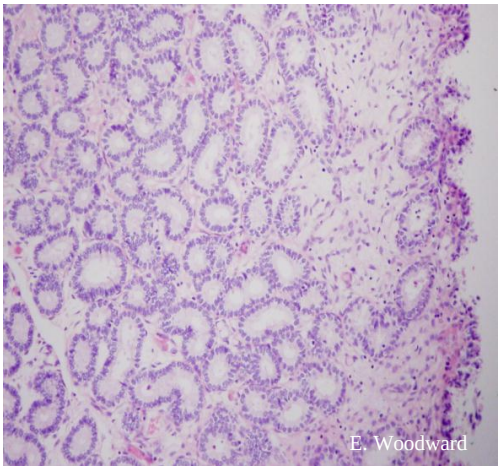
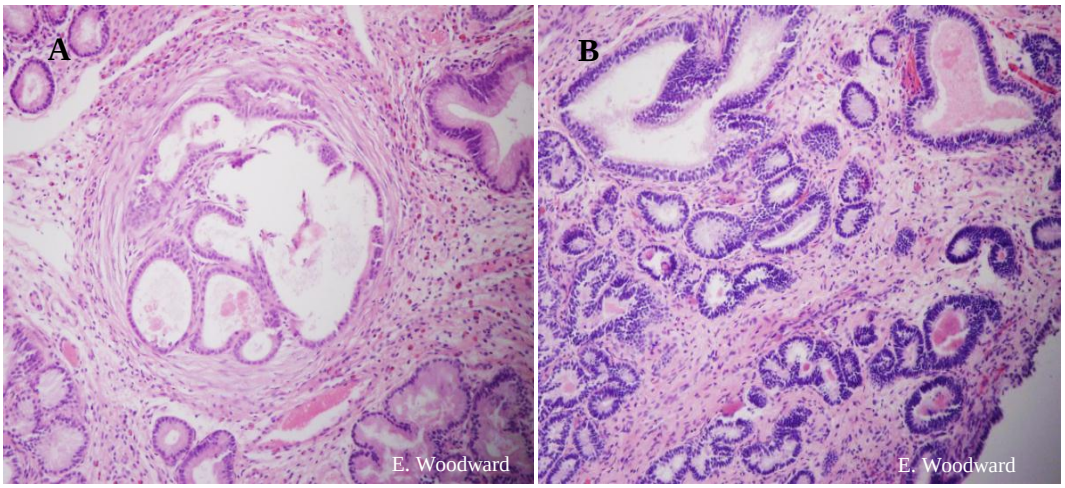


Figure 1.3: Endometrial biopsies from a grade III mare with a fibrotic nest (A), and severe degenerative changes (B) (×400 magnification).



1.2 Uterine Defense Mechanisms

1.2.1 *The susceptible and resistant mare*

Breeding induced endometritis is a normal physiological reaction, as it is believed that an inflammatory response is necessary for the effective removal of bacteria and excess spermatozoa introduced into the uterus (Troedsson 2006). In a healthy uterus, the inflammation subsides within 48 hours; however a subset of mares fails to clear the

inflammation in a timely fashion. Hughes and Loy observed a disparity in the ability to resolve uterine inflammation between young, fertile mares, and older, sub-fertile mares after an intrauterine challenge with *Streptococcus zooepidemicus* (Hughes & Loy 1969). The authors suggested that the mares less successful in clearing their inflammation had an alteration in their normal uterine defense mechanism. Persistent endometritis impairs fertility and threatens the viability of the conceptus, which migrates to the uterus by day 5 after breeding (Oguri & Tsutsumi 1972, Betteridge *et al.* 1982, Troedsson *et al.* 1995a, Troedsson 1999). Literature suggests that an estimated 10 to 15% of broodmares retain fluid 24-36 hours after breeding (Zent *et al.* 1998), and persistent endometritis has been cited as a leading reproductive health concern in equine veterinary practice (Traub-Dargatz *et al.* 1991).

Predisposition to persistent endometritis has been investigated and attributed (in part) to multiple factors. Advanced age has been associated with an altered systemic immune response in horses and other animal species, and it has been observed that age is correlated to susceptibility to persistent endometritis (Ricketts & Alonso 1991, Carnevale & Ginther 1992, Zent *et al.* 1998, Barbacini *et al.* 2003). Internal and external conformation of the reproductive organs has been associated with susceptibility to persistent endometritis. Mares with poor perineal conformation had lower conception rates than normal mares (Hemberg *et al.* 2005). Furthermore, the position of the uterus within the abdominal cavity was found to be relevant in the ability to clear inflammation when researchers found that mares less successful in clearing radiocolliod had a "pendulous" uterus dropping below the pelvic brim (LeBlanc *et al.* 1994, LeBlanc *et al.* 1998). In addition, it has been suggested that endometrial pathologies are correlated to

susceptibility to persistent infectious endometritis (Troedsson *et al.* 1993a). Finally, the condition of the uterus before challenge has been associated with susceptibility to persistent endometritis in several studies. Mares with excess fluid retention during diestrus were more likely to have problems with uterine fluid retention after breeding (Brinsko *et al.* 2003), and mares with a positive uterine culture prior to breeding had lower pregnancy rates than mares with negative uterine cultures (Riddle *et al.* 2007).

Since the diagnosis of persistent endometritis is made after the fact, methods to predict susceptibility to persistent endometritis are often limited to the mares' clinical histories. Identifying mares susceptible to persistent endometritis prior to breeding to proactively treat before the onset of persistent endometritis is important to increase the chance of a successful pregnancy. Mares have been classified as susceptible to persistent endometritis based on reproductive history, fluid retention after insemination (Brinsko *et al.* 2003), failure to clear a bacterial or spermatozoa challenge (Troedsson *et al.* 1993a, Troedsson *et al.* 1993b, Alghamdi *et al.* 2005b, Woodward *et al.* 2011a), and endometrial pathology (Woodward *et al.* 2011b).

1.2.2 *The humoral and cellular immune responses*

Immunoglobulins in the uterus were suggested to be involved with the development of persistent endometritis. Higher amounts of IgA, IgG, and IgG(T) were found in the uterine fluid of mares with impaired resistance to persistent endometritis when compared to normal mares (Asbury *et al.* 1980). A larger study found similar results with IgA levels, but did not detect a difference with levels of IgG or IgG(T) (Mitchell *et al.* 1982). Another study found that mares with positive uterine cultures had

increased levels of IgA, (but not IgG or IgG(T)) in their uterine secretions compared to mares with negative cultures (Williamson *et al.* 1983). After a bacterial challenge, Troedsson *et al*. observed a decline in uterine levels of IgG and complement C3 for the first 24 hours post challenge (indicating a uterine response and opsionization of sperm and pathogens) in mares susceptible or resistant to persistent endometritis. However, the researchers did not detect a difference between the two groups of mares, and therefore concluded that although the adaptive immune system does play a role in endometritis, it is unlikely that susceptibility is primarily caused by a defect in this mechanism (Troedsson *et al.* 1993d).

Polymorphonuclear neutrophils (PMNs) are white blood cells that respond to inflammation and function to remove pathogens through two mechanisms. Their primary mechanism of action is to bind and engulf microbes through phagocytosis. A second mechanism is through the release of neutrophil extracellular traps (NETs), composed of extensions of DNA and histones with antimicrobial peptides. The NETs are extended towards a target and capture the target (Brinkmann *et al.* 2004, Amulic & Hayes 2011).

Initial studies hypothesized that mares susceptible to persistent endometritis had defective uterine PMNs. Uterine PMNs from susceptible mares were found to have decreased phagocytic ability (Cheung *et al.* 1985, Watson *et al.* 1987) and overall functionality (Liu *et al.* 1985) when compared to PMNs from resistant mares.

The complement system and opsonization of cells has been examined to study how the uterus recognizes and removes sperm and bacteria. Complement plays an important role in effective phagocytosis by PMNs by marking pathogens for phagocytosis (Asbury *et al.* 1984, Hakansson *et al.* 1993). Uterine secretions from sub-

fertile mares were worse at promoting bactericidal activity than secretions from resistant mares (Watson *et al.* 1987). Other researchers confirmed this finding when they observed that the PMNs of susceptible mares were able to become fully functional in the right environment (contrary to previous reports that PMNs from susceptible mares have decreased functionality). The authors concluded that susceptible mares have an impaired intrauterine opsonizing ability (Troedsson *et al.* 1993c)

1.2.3 The role of seminal plasma

Seminal plasma modifies the uterine inflammatory response to spermatozoa. Activation of the complement cascade was inhibited by seminal plasma (Troedsson *et al.* 2000). Complement is an important opsonin, and opsonization of spermatozoa was reduced in the presence of seminal plasma *in vitro* (Alghamdi *et al.* 2004, Troedsson *et al.* 2005). In addition, the binding of PMNs to opsonized spermatozoa, and subsequent phagocytosis was reduced in the presence of seminal plasma, further illustrating the protective properties that seminal plasma has on spermatozoa (Troedsson *et al.* 2005). Pregnancy rates in mares with induced endometritis prior to breeding were higher (77%) in mares bred with spermatozoa and seminal plasma compared to mares bred with spermatozoa and semen extender (5%) (Alghamdi *et al.* 2004). It has been suggested that the uterine defense mechanism selectively targets dead spermatozoa, which may be due to specific proteins in the seminal plasma (Alghamdi & Foster 2005a, Doty *et al.* 2011). Seminal proteins were fractioned, and one protein was found to suppress PMN binding to viable sperm, while another was found to promote binding to snap frozen (killed) sperm (Troedsson *et al.* 2005). Researchers identified the PMN suppressing protein in equine

semen as cysteine-rich secretory protein-3 (Doty *et al.* 2011), which was previously linked to fertility (Hamann *et al.* 2007, Novak *et al.* 2010).

1.2.4 *Cytokines and the innate immune response*

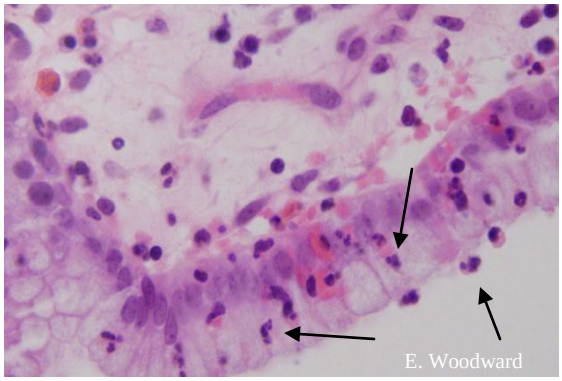
Inflammatory cytokines are cell signaling molecules associated with the recognition and resolution of the inflammatory response. They are produced by many cells types acting locally in an autocrine or paracrine fashion, and systemically in an endocrine function (Feghali & Wright 1997). Pro-inflammatory cytokines initiate inflammation, and once produced, a cascade of inflammatory events occurs (Kuby 1992, Parham 2005). A timely response to pathogens is important for an effective resolution of inflammation and preservation of tissue (Schroder *et al.* 2004). There is a delicate balance between the pro and anti-inflammatory response, as anti-inflammatory cytokines modulate the pro-inflammatory cytokines (Parham 2005, Hackett *et al.* 2008).

Two of the primary cytokines involved with mediating acute inflammation after injection with lipopolysaccharide are interleukin (IL)1 and tumor necrosis factor (TNF) (Feghali & Wright 1997). IL1 is released by several types of cells, including inflammatory and epithelial cells, and targets cells to induce the release of other cytokines and histamine, the transcription of inducible nitric oxide synthase (iNOS), and the production of prostaglandin E_2 (PGE_2) (Kuby 1992). It is regulated by the IL1 receptor antagonist (IL1RA) which acts through competitive binding to the IL1 receptor (Feghali & Wright 1997). Furthermore, the IL1 system is involved with both pathologic states as well as normal reproductive function (Takehara *et al.* 1994, Van der Hoek *et al.* 1998, Martoriati *et al.* 2003, Gerard *et al.* 2004). In addition to IL1RA, the inflammatory

modulating cytokine, IL10, is essential for the proper function of the immune response, and deficiencies in IL10 can lead to tissue damage (Cyktor & Turner 2011).

TNF (α and β) induces apoptosis in infected and tumor cells, and can act to induce the synthesis of PGE_2 directly or indirectly through the induction of IL1. In addition to inducing the production of acute phase proteins by the liver, both IL1 and TNFα stimulate IL6 production, which acts to modulate TNFα and IL1 during the early phase of inflammation. IL6 has both pro- and anti-inflammatory properties (Feghali & Wright 1997). It has protective roles in early acute inflammation and shock through the modulation of other pro-inflammatory cytokines (Barton & Jackson 1993, Xing *et al.* 1998, Diao & Kohanawa 2005), and the induction of inflammatory modulating cytokines, such as IL1RA (Tilg *et al.* 1994). However, in addition to the resolution of acute inflammation, IL6 and its receptor have been suggested to have implications in the transition from innate to acquired immunity and the persistence of chronic inflammation (Kishimoto *et al.* 1995, Atreya *et al.* 2000, Jones 2005). Another inflammatory cytokine, IL8, is released by macrophages and endothelial cells and acts as a chemoattractant for PMNs (Liu *et al.* 1997). In addition to its chemoattractive properties, IL8 upregulates the expression of cell surface adhesion molecules to aid in the adherence to endothelial cells and diapedesis of the PMNs through blood vessel walls (Figure 1.4) (Atreya *et al.* 2000). Interferon gamma (IFNγ) is a type II interferon produced by activated T cells and natural killer cells. It is distinct from the type I interferons (which are primarily antiviral) in that it stimulates effector functions of mononuclear phagocytes, and activates macrophages to remove pathogens (such as mycobacteria) (Feghali & Wright 1997).

Figure 1.4: Neutrophil migration through the equine endometrial epithelium.



Cytokine mRNA expression during the uterine inflammation has been studied in the cow (Gabler *et al.* 2009, Gabler *et al.* 2010), however, few studies have focused on the mare. In one experiment, an increase in mRNA levels IL1, IL6, and TNFα was detected in all mares 24 hours after artificial insemination, but susceptible mares had higher basal levels of the cytokines than resistant mares (Fumuso *et al.* 2003b). The same researchers subsequently found increased mRNA expression of IL8 and lower expression of IL10 in susceptible mares compared to resistant mares 24 hours after insemination (Fumuso *et al.* 2006, Fumuso *et al.* 2007). However, other authors were unable to find a significant IL8 response to insemination in reproductively normal pony mares 24 hours after insemination (Nash *et al.* 2008, Nash *et al.* 2010).

Endometritis induced by bacterial challenge has been used as a model to investigate endometrial cytokine expression. Endometrial mRNA expression of TNFα, IL1β, IL8, and IL10 were all found to be upregulated in response to a diestrus intrauterine inoculation of a high dose of *Escherichia coli* as early as 3 hours after challenge in normal mares (Christoffersen *et al.* 2010). Although bacterial and breeding induced endometritis are studied separately for experimental purposes, separating the two in practical settings is almost impossible. Natural breeding introduces bacteria into the

uterus, and even the best efforts for a sterile environment during artificial insemination offer the risk of bacterial contamination into the uterus. Reproductively sound mares should be able to rapidly resolve inflammation caused from either stimulus, however, there is a difference in the recognition of gram negative bacteria and gram positive bacteria (Aderem and Ulevitch 2000), and it is possible that different inflammatory pathways are activated for spermatozoa and for bacteria. Finally, the resolution of inflammation on a cytokine level may differ due to the nature of bacterial versus spermatozoa challenges, as bacteria continue to reproduce in the reproductive tract throughout the clearance process, while spermatozoa may stimulate the immune response only once at the time of insemination.

1.2.5 *Physical defenses*

In addition to cytokine signaling and PMN recruitment, the uterus acts mechanically to expel excess semen and debris associated with breeding. As activated PMNs pass through the endometrium, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is released from the epithelial cells and PMNs, causing myometrial contractions (Troedsson 1999). In addition, oxytocin release and subsequent myometrial activity has been associated with stallion calls and other breeding stimuli (Madill *et al.* 2000), aiding in uterine clearance. Troedsson and Liu (1991) observed that mares susceptible to persistent uterine infection had a delay in clearing ^{51}Cr microspheres from the uterus when compared to normal, resistant mares. The study demonstrated a failure in the physical ability of the uterus to expel uterine contents (Troedsson & Liu 1991). Efficient uterine expulsion of fluid and debris involves synchronized contractions of longitudinal and circular myometrial tissues

(Hirsbrunner *et al.* 2006, Hirsbrunner *et al.* 2010). Using electrodes, Troedsson *et al.* observed that mares susceptible to persistent endometritis had a decrease in electrical patterns in duration, frequency, synchronization, and intensity when compared to resistant mares (Troedsson *et al.* 1993b). Failure of uterine clearance was further investigated using scintigraphy when other researchers observed that resistant mares began to clear radiocolliod as early as 30 minutes after challenge (while the susceptible mares were slower to respond), and by 2 hours, the susceptible mares retained more radiocolloid than the resistant mares (LeBlanc *et al.* 1994). Based on these studies, it was concluded that myometrial contractility defects may be a major contributor in the pathogenesis of persistent endometritis.

1.2.6 *Nitric oxide*

Nitric oxide (NO) is a potent cell signaling molecule occurring in many body systems in physiological and pathological states (Hou *et al.* 1999), and has both protective and deleterious effects in the body (Tripathi 2007, Angulo *et al.* 2010, Eleftheriadis *et al.* 2010, Gupta *et al.* 2010, Zanetti *et al.* 2010). NO is synthesized by nitric oxide synthase (NOS) in many cell types, including macrophages, neutrophils, hepatocytes, epithelial and endothelial cells (Li *et al.* 2010). Three types of NOS have been identified. Endothelial NOS (eNOS or NOS3), is present in endothelial tissue, and is involved with regulation of vasodilatation (Rosselli *et al.* 1998, Tripathi 2007). Neuronal NOS (nNOS or NOS1) is present in nervous tissue and produces NO to serve as a neurotransmitter. Similar to eNOS, nNOS produces NO constitutively in low amounts, and is active during normal biological events (Rosselli *et al.* 1998, Gensert & Ratan

2006). Unlike eNOS and nNOS, iNOS produces NO in large amounts (Rosselli *et al.* 1998, Saxena *et al.* 2000, Tripathi 2007, Li *et al.* 2010). During inflammation, inflammatory cytokines such as IL1β and IFNγ initiate a cascade of events leading to the transcription of iNOS (Green *et al.* 1994, Rosselli *et al.* 1998, Tripathi 2007). The increased NO produced by iNOS serves as an aid in the removal of pathogens but can also be cytotoxic (MacMicking *et al.* 1997, Tripathi 2007). NO is also a known smooth muscle relaxer, and myometrial tissue *in vitro* was unable to respond to electrical stimulus in the presence of NO (Liu *et al.* 1997). In addition, mares susceptible to PBIE had increased iNOS activity and NO production when compared to resistant mares 13 hours after insemination (Alghamdi *et al.* 2005b). The authors suggested that contractility deficiencies in susceptible mares between 6 and 19 hours after insemination (Troedsson *et al.* 1993b) may be associated with increased NO production (Alghamdi *et al.* 2005b).

1.2.7 *Summary of the development of endometritis*

A local uterine immune response has been documented in response to spermatozoa, semen, and bacteria (Troedsson *et al.* 2001). For experimental purposes, endometritis has been examined using both spermatozoa and bacterial models. However, it is not possible to separate inflammation resulting from bacteria and spermatozoa in breeding situations, as bacteria are likely to be unintentionally incorporated into the uterus during breeding. However, in both cases, healthy mares should be able to resolve the endometritis. This inflammation is believed to be initiated by the activation of complement, which in turn leads to the activation of inflammatory cytokines (Kuby 1992, Troedsson *et al.* 2001, Parham 2005). The release of inflammatory cytokines triggers the

recruitment of PMNs through the endometrium and into the uterine lumen (Kotilainen *et al.* 1994, Troedsson 1999). Activated PMNs bind to spermatozoa targeted by opsonins (such as complement factor C3b), or capture spermatozoa and pathogens with NETs (Dahms & Troedsson 2002, Alghamdi *et al.* 2004). The activation of PMNs triggers the production and release of $PGF_{2\alpha}$, leading to myometrial contractions which aid in the expulsion of excess semen and debris associated with breeding (Troedsson 1999). Finally, other hormones with ecobolic effects (such as oxytocin) may be released as a result of the teasing and breeding process, contributing to uterine clearance (Figure 1) (Madill *et al.* 2000). Given previous research, a proposed diagram of the development of breeding induced endometritis can be drawn, however questions remain as to specifics in cytokine mRNA expression and NO production (Figure 1.5)

1.3 Clinical Signs and Diagnosis of Endometritis

Endometritis can be associated with bacterial infections, or can occur in response to semen, but a common denominator is the inflamed state of the endometrium determined by the presence of neutrophils and fluid accumulation (Troedsson 2006). Clinical signs are rare, however, vaginal discharge, a shortened luteal phase, and decreased fertility can be observed. Uterine cytological exam is accomplished using several techniques such as uterine swab (double guarded or single guarded), brush, biopsy or low volume lavage (Ball *et al.* 1988, Card 2005, Kasimanickam *et al.* 2005, Nielsen 2005, Cocchia *et al.* 2012). When compared to an endometrial swab, cytological exam from an endometrial biopsy was found to be the most sensitive method to determine the presence of endometritis (Nielsen 2005). Choosing a method is the option

of the clinician, as all techniques are accepted, however, obtaining the samples (regardless of technique) requires adequate training and knowledge to collect and process the samples, as any of the methods can yield a false negative if not conducted properly. There are several accepted methods to evaluate uterine cytological samples (Table 1.1), and these methods are used in combination with using other diagnostic tools to diagnose inflammation (Card 2005).

Uterine fluid retention is assessed by ultrasonography (McKinnon *et al.* 1988). Like uterine cytological exam, there are several methods for evaluating uterine fluid retention. Transrectal ultrasonography has greatly advanced the treatment and diagnosis of fluid retention and endometritis. Retained uterine fluid has been graded based on echogenicity and volume. Although some fluid is expected immediately after breeding, more than 2 cm of fluid between 6 and 36 hours after breeding, or fluid that is high in echogenicity (indicative of purulent components and infection) is considered problematic and warrants treatment (Troedsson 1997). However, caution should be exercised when attempting to quantify uterine fluid accumulation, as manipulation of the reproductive tract will disperse fluid and alter results. Both uterine cytological exam and evaluation for uterine fluid are good methods for assessing inflammation, however combining both parameters is a practical way to more accurately assess the state of the endometrium (Card 2005).

Figure 1.5: Proposed sequences of events occurring after breeding in susceptible and resistant mare based on current literature. Yellow text refers to resistant mares, orange text to susceptible mares, white text to both resistant and susceptible mares, and blue text is unknown.

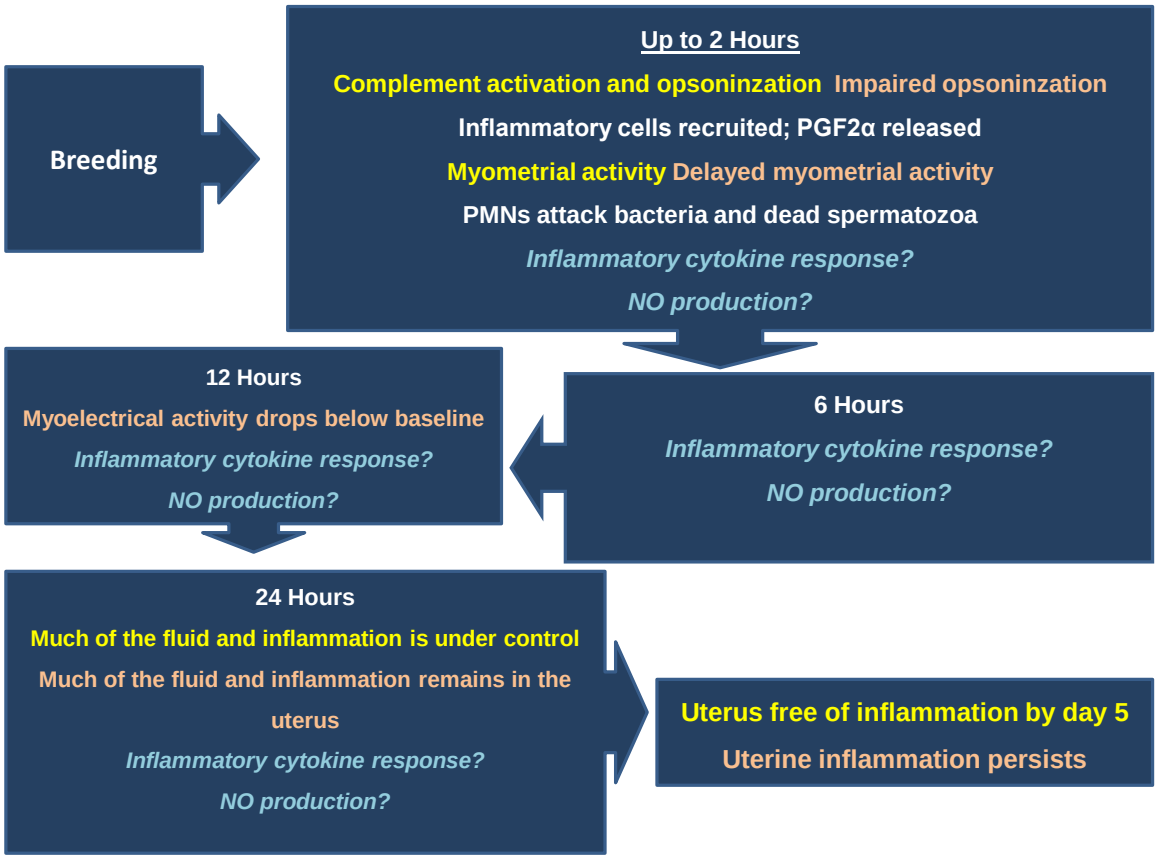


Table 1.1: Quantitative methods for the interpretation of equine endometrial cytology samples (from Card 2005).

>1 neutrophil in five fields (×240)	Knudsen [11]	1964
Ratio of neutrophils to epithelial cells	Digby and Ricketts [12]	1978
Ratio of epithelial cells to neutrophils >10:1	Asbury [13]	1982
>1 neutrophil per high powered field	Asbury [14]	1984
Neutrophils to epithelial cells eight fields	Couto and Hughes [15]	1984
>5 neutrophils in 10 fields	Brook [16]	1985
Ratio of neutrophils to epithelial cells	La Coeur and Sprinkle [17]	1986
<15 endometrial cells to 1 neutrophil	Ley [18]	1986
≥3–10% of cells are neutrophils	Crickman and Pugh [19]	1986
≥2% of cells are neutrophils	Ball et al [20]	1988
≥1 neutrophil per field (×400)	Purswell [21]	1989
>0.5% neutrophils	Ricketts and Mackintosh [22]	1989

1.4 Treatments

There are a variety of treatment strategies available for resolving persistent endometritis, including administration of mucolytics to break up mucus associated with bacterial infection, ecbolics to clear fluid, antibiotics to kill bacteria, uterine lavage to physically flush the uterine contents, and surgical correction of the reproductive tract to assist in preventing the entrance of bacteria. These treatments are often used in combination with variations in dose, frequency, duration of treatment, and administration route, and are administered both proactively and reactively (Combs *et al.* 1996, Watson 2000, Bliss & Campbell 2002, Hurtgen 2006, Liu & Troedsson 2008, LeBlanc 2010).

The use of corticosteroids to proactively treat problem mares has also been proposed as an effective treatment strategy. Pregnancy rates were improved in mares treated with 5 doses of predinisolone acetate in mares with a history of fluid retention

after breeding (Dell' Aqua *et al.* 2006). Furthermore, oral administration of 200 mg of dexamethasone twice daily for 5 days before breeding improved pregnancy rates in sub-fertile mares (Morris & Eden 2008). When condensing treatments into one dose, Bucca *et al*. (2008) saw a reduction in clinical signs of persistent endometritis with iv administration of 50 mg of dexamethasone at the time of breeding, and concluded that a single dose of 50 mg dexamethasone is both safe and effective for the treatment of persistent endometritis (Bucca *et al.* 2008). Corticosteroids are known to suppress the expression of pro-inflammatory cytokines in other species and body systems (Hodge *et al.* 1999, Elenkov 2004). While the anti-inflammatory effect of corticosteroids may modulate the breeding-induced inflammation, adverse effects on the killing capacity of PMNs has been reported (Dell' Aqua *et al.* 2006). However, other researchers reported that mares treated with 50 mg dexamethazone had no effect on PMN migration or phagocytosis (Bucca *et al.* 2008, Ohman *et al.* 2011).

Mycobacterial cell wall extract (MCWE) is a potent immune stimulator and has been used to stimulate the immune response to treat cancer (Filion & Phillips 2001, Yuksel *et al.* 2011), respiratory disease, and as a vaccine adjuvant (Berinstein *et al.* 1991). MCWE has been used as a proactive treatment for persistent endometritis, and was found to decrease the number of mares with endometritis after challenge with *streptococcus zooepidemicus* (Rogan *et al.* 2007). Pregnancy rates in mares bred during their foal heat cycle were improved after treatment with MCWE (Fumuso *et al.* 2003a). In addition, when investigating the uterine inflammatory response to breeding, MCWE was shown to decrease levels of pro-inflammatory cytokines in susceptible mares towards levels of resistant mares 24 hours post breeding (Fumuso *et al.* 2003b, Fumuso *et*

al. 2007). The authors concluded that treatment with MCWE was beneficial in terms of returning the uterine inflammatory cytokine response to homeostasis and improving fertility, however, the mechanism of MCWE needs further investigation to understand the timing of inflammatory events occurring after treatment and challenge.

1.5 Conclusion

The uterine environment is one of complex interactions, requiring a delicate balance of recognizing and responding to inflammatory stimuli to maintain functionality. The position and conformation of the mare reproductive tract can lead to challenges in fertility which can be diagnosed and addressed in a variety of ways. Although there are many treatment options for the treatment of PBIE, it is crucial to identify mares susceptible to PBIE prior to challenge so that proactive treatments can be limited to only the mares that will need them, and administered to those mares more likely to have persistent inflammation. In addition, a better understanding of the uterine inflammatory pathways and how current treatment strategies affect these pathways is important for improving treatment regimes. Therefore, the first objectives of the following experiments are to investigate the relationship of endometrial quality with age and fluid retention after breeding, and to observe how mares change their susceptibility from one season to the next. A second experiment investigates cytokine mRNA expression in resistant and susceptible mares within the first 24 hours after breeding, and given the results from that experiment, an additional study examines the effect of immunomodulation on inflammatory cytokine mRNA expression in susceptible mares 6 hours after breeding. Lastly, a final experiment assesses intrauterine NO production and accumulation in

resistant and susceptible mares within the first 24 hours after breeding, and the effects of immunomodulation on NO production and accumulation in the uterus 6 hours after breeding.

CHAPTER 2

Susceptibility to persistent breeding induced endometritis: relationship to endometrial biopsy score and age, and variations between seasons.

Woodward, E[a*], Christoffersen, M[b], Campos, J[a], Squires, E.L[a], Troedsson, M.H.T.[a]

[a]The Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, 40546-0099, USA

[b]Department of Large Animal Sciences, Veterinary Reproduction and Obstetrics, University of Copenhagen, Dyrlaegevej 68, DK-1870 Frederiksberg C, Denmark

Accepted for publication Theriogenology, April, 2012

2.1 Summary

The objectives were to: 1) investigate the associations of age and endometrial biopsy score with uterine fluid retention after insemination; and 2) determine if a strict classification of susceptibility to persistent breeding induced endometritis (PBIE) based on biopsy score, endometrial cytology, and fluid retention after inseminations, is consistent over subsequent breeding seasons. In Experiment 1, 57 mares were inseminated with 10^9 freeze-killed sperm during estrus and evaluated for uterine fluid retention 48 and 96 hours after insemination. Comparisons were made between fluid retention and biopsy score or age. In Experiment 2, a subset of 14 mares was classified for susceptibility to PBIE in 2 subsequent breeding seasons. Biopsy score and age were associated with fluid retention ($P < 0.001$). In addition, age was related to biopsy score (P

< 0.001). Of the mares examined for susceptibility, 36% (5 of 14) changed status during subsequent seasons. 3 mares changed to a more severe classification (intermediate to susceptible, or resistant to intermediate), whereas 2 mares changed to a less severe classification (susceptible to intermediate).

2.2 Introduction

Transient breeding-induced endometritis is a normal event in the immediate hours after breeding, and it is believed that an inflammatory response is necessary for effective removal of bacteria and excess spermatozoa introduced into the uterus (Troedsson 2006). In a healthy uterus, inflammation is cleared well before the fertilized oocyte descends from the oviduct into the uterine lumen, 5 days after ovulation and conception (Oguri & Tsutsumi 1972, Betteridge *et al.* 1982). A timely resolution of breeding induced inflammation is necessary in order to provide an environment that is compatible with pregnancy (Troedsson 1999). Using a bacterial challenge with *Streptococcus zooepidemicus*, Hughes and Loy reported that whereas most mares were able to clear bacteria from the uterus, a subpopulation of predominantly older mares was not (Hughes & Loy 1969). The authors suggested that these mares had an alteration in the normal uterine defense mechanism. Persistent endometritis can be associated with bacterial infections, or can occur in response to semen. In most cases, breeding induced endometritis is the result of the combined exposure to semen and bacteria, since bacteria can enter the uterus during both natural mating and artificial insemination. Therefore, it is not possible to designate the inflammatory agent as either sperm or bacteria (Troedsson 1995a). Regardless of the cause of endometritis, a common denominator is the inflamed

state of the endometrium, manifested by the presence of neutrophils and fluid accumulation (Troedsson 2006), although reproductively healthy mares should resolve the inflammation efficiently.

Age has been associated with an altered systemic immune response in horses and other animal species, and it has been observed that age is a factor in mares prone to PBIE (Ricketts & Alonso 1991, Carnevale & Ginther 1992, Zent *et al.* 1998, Barbacini *et al.* 2003). In addition, other factors have been linked to sub-fertility associated with PBIE, including poor perineal conformation (Hemberg *et al.* 2005), excess fluid retention during estrus prior to breeding (Brinsko *et al.* 2003), a defect in myometrial contractility (Troedsson *et al.* 1993b), uterine position within the abdominal cavity (LeBlanc *et al.* 1998), and a positive uterine culture prior to breeding (Riddle *et al.* 2007). Troedsson *et al.* observed that mares susceptible to persistent uterine infection delayed clearing ^{51}Cr microspheres from the uterus when compared to normal, resistant mares; that study demonstrated a failure in the physical ability to expel uterine contents (Troedsson & Liu 1991). Using scintigraphy, Leblanc *et al.* (1994) confirmed that delayed uterine clearance was a consistent finding in mares susceptible to persistent endometritis (LeBlanc *et al.* 1994). Although scintigraphy and microspheres are useful methods for studying PBIE, they are not practical as diagnostic tools in a clinical setting. Since the diagnosis of PBIE is made after the fact, methods to predict susceptibility to PBIE are often limited to the mares' clinical histories.

Endometrial biopsy score is an accepted marker of uterine health and predicted fertility (Ludwig *et al.* 2001, Schlafer 2007), and it has been suggested that endometrial pathologies are correlated to susceptibility to persistent infectious endometritis

(Troedsson *et al.* 1993a). However, the number of observations in previous studies was relatively small and it is not clear if susceptibility to PBIE is maintained during subsequent breeding seasons.

The objectives of this study were to: 1) investigate associations of age and endometrial biopsy score with uterine fluid retention after insemination; and 2) determine if a strict classification of susceptibility to PBIE (based on biopsy score, endometrial cytology, and fluid retention) after insemination is consistent over subsequent breeding seasons. Based on previous data, it was hypothesized that mares with biopsy scores of IIB and III (indicating more degenerative changes, [21]) are more prone to uterine fluid retention after breeding than mares with scores I and IIA. Furthermore, it was hypothesized that mares retain their status or exhibit an increase in susceptibility to PBIE during subsequent seasons. No attempt was made to implicate sperm, bacteria, or a combination of the two, as the cause of PBIE, or to associate the grade of uterine fluid with susceptibility to PBIE.

2.3 Materials and Methods

2.3.1 Animals

Mares of mixed breeds and ages (2 to 27 years) were kept on pasture (and fed supplemental grain and hay), and provided with water and salt *ad libitum*. The mares were donations to the University research herd, and their reproductive histories were mostly unknown. Semen was collected from 3 stallions with housing conditions similar to that of the mares. Both experiments were approved by the University of Kentucky's IACUC (protocol numbers 2009-0455 and 2009-0602).

2.3.2 Preparation of sperm for insemination

Freeze-killed sperm were prepared by removing the seminal plasma from ejaculates using centrifugation (1000 × g for 10 minutes), then re-suspending the sperm pellet in EquiPro™ milk based extender (Minitube, Verona, WI, USA) in aliquots of 10^9 sperm in 30 mL of extender, followed by at least 2 freeze (-20 °C)/thaw cycles.

2.3.3 General experimental procedure

Mares were observed regularly using transrectal ultrasonography, and estrus was determined based on uterine edema, a relaxed cervix, and at least one follicle 35 mm or larger in diameter in the absence of a *corpus luteum*. Prior to inseminations, mares were evaluated for the presence of intrauterine polymorphonuclear neutrophils, (PMN) using a cytobrush (Minitube) or low volume lavage to obtain samples, and for intrauterine bacteria using a double-guarded swab (Minitube) to obtain samples. Inflammation was defined as more than 2 PMNs per 5 fields at ×400 magnification. Only mares with negative cytology and culture prior to insemination were considered for the experiments. If a mare had a positive culture and/or cytology prior to insemination, she was excluded for that cycle, treated, and re-entered the study during a subsequent cycle. For all inseminations, mares were challenged with an intrauterine inoculation of 10^9 freeze-killed stallion sperm in 30 mL of milk based semen extender (Minitube). During diestrus, endometrial biopsies (approximately 300 mg of tissue) were acquired using an alligator jaw biopsy instrument, then fixed in 10% formalin, sectioned at 5 μm and stained with hematoxylin and eosin. Each biopsy was examined for periglandular fibrosis,

inflammatory cells, glandular distribution and lymphatic lacunae, then graded according to Kenny and Doig (Kenney & Doig 1986). Two experiments were conducted to investigate the specific aims. Experiment 1 aimed to compare age and endometrial biopsy score to uterine fluid retention 48 and 96 hours after insemination. Experiment 2 aimed to determine if a strict classification of susceptibility to PBIE based on biopsy score, endometrial cytology, and fluid retention after inseminations, was consistent over subsequent breeding seasons.

2.3.4 Experiment 1: uterine fluid retention after breeding

Fifty-seven mares (aged 2 to 27 years) were evaluated for uterine fluid retention by transrectal ultrasonography 48 and 96 hours after insemination. Fluid retention was defined as having > 2 cm of intrauterine fluid 48 hours after insemination, or the presence of any fluid 96 hours after insemination.

For statistical comparisons on effect of age, mares were allocated into 3 groups (2 to 8 years, 9 to 16 years, ≥ 17 years). Using SigmaStat© (Systat Software, Inc., Chicago, IL, USA), statistical analyses were made using a Chi-square test comparing biopsy score, uterine fluid retention, and age. Individual comparisons of each biopsy grade and age group with the presence or absence of uterine fluid were made using a Fischer's exact test. Significance was set at $P < 0.05$.

2.3.5 Experiment 2: susceptibility to PBIE over subsequent seasons

Fourteen mares (aged 5 to 27 years) were examined during 2 subsequent breeding seasons for susceptibility to PBIE based on diestrus endometrial biopsy score and the

results of a sperm challenge. Based on the results from Experiment 1, mares with a score of I or IIA were considered potentially resistant to PBIE, whereas those with scores of IIB or III were potentially susceptible to PBIE.

After selection as potentially resistant or susceptible to PBIE, mares were inseminated with freeze-killed sperm during estrus. Potentially susceptible mares were evaluated 96 hours after insemination and were confirmed as susceptible if they had: 1) positive cytology, and 2) uterine fluid accumulation. Potentially resistant mares were assessed 48 hours after breeding, and those with: 1) negative cytology/culture, and 2) no uterine fluid accumulation, were confirmed as resistant mares. Mares not meeting the criteria for classification as susceptible or resistant under the conditions of this study were considered intermediate. A comparison was made examining changes in classification from one year to the next (reported as a percentage).

2.4 Results

2.4.1 Experiment 1

Of the 57 mares evaluated for uterine fluid retention after breeding, 19 had a biopsy score of I or IIA (4 I; 15 IIA), and 38 with IIB or III (16 IIB; 22 III). Nine mares were in age group one, 22 in group 2, and 26 in group 3. One of the mares in age group one had a biopsy score of I, 7 with a score of IIA, and one with a score of IIB. Three of the mares in group 2 had a score of I, 8 had a score of IIA, 6 had a score of IIB, and 5 had a score of III. Nine of the mares in age group 3 had a score of IIB, and 17 had a score of III. Two mares with grades IIA and III did not retain fluid 48 hours after insemination, but did have fluid 96 hours after insemination.

Comparisons were made between biopsy grades and uterine fluid retention at the 48 and 96 hours time points (Fig. 2.1). Forty-eight hours after insemination, 13% (2 of 15) of the grade IIA mares, 75% (12 of 16) of the grade IIB mares, and 86% (19 of 22) of the grade III mares retained fluid. Ninety-six hours after insemination, 7% (1 of 15) of the grade IIA mares, 69% (11 of 16) of the grade IIB mares, and 91% (20 of 22) of the grade III mares retained fluid. None of the mares with a grade I biopsy (0/4) retained fluid 48 or 96 hours after insemination. For both time points, there was no difference in intrauterine fluid retention when comparing biopsy grades I to IIA or grades IIB to III. However, 48 hours after insemination, there was an increase in incidence of uterine fluid retention for grades IIB and III when comparing grade I to IIB ($P = 0.014$) and III ($P = 0.002$), and when comparing grade IIA to IIB ($P < 0.001$) and III ($P < 0.001$). In addition, 96 hours after insemination, there was an increase in incidence of uterine fluid retention for grades IIB and III when comparing grade I to IIB ($P = 0.026$) and III ($P = 0.001$), and when comparing grade IIA to IIB ($P = 0.002$) and III ($P < 0.001$)

Comparisons were made between age group and uterine fluid retention at 48 and 96 hours (Fig. 2.2). None of the mares in group one (0/9) retained fluid 48 or 96 hours after insemination. Forty-five percent (10/22) of the group 2 mares retained fluid 48 hours after insemination, and 36% (8/22) retained fluid 96 hours after insemination. Eighty-eight percent (23/26) of group 3 mares retained fluid 48 and 96 hours after insemination. For both time points, there was no difference in intrauterine fluid retention when comparing age group one to group 2. However, 48 hours after insemination, there was an increase in incidence of uterine fluid retention for age group 3 when compared to age group one ($P < 0.001$) and age group 2 ($P = 0.001$). In addition, 96 hours after

insemination, there was an increase in incidence of uterine fluid retention for age group 3 compared to age group one ($P < 0.001$) and to age group 2 ($P = 0.001$).

Based on these results, Chi Square analysis was performed with biopsy scores I and IIA combined into one group, and IIB and III into another. Both biopsy score and age were related to fluid retention ($P < 0$ .001). In addition, the biopsy score of mares was related to age ($P < 0.001$).

Fig. 2.1: Biopsy score and intrauterine fluid in mares 48 and 96 hours after insemination. Columns without a common superscript differed ($P < 0.05$).

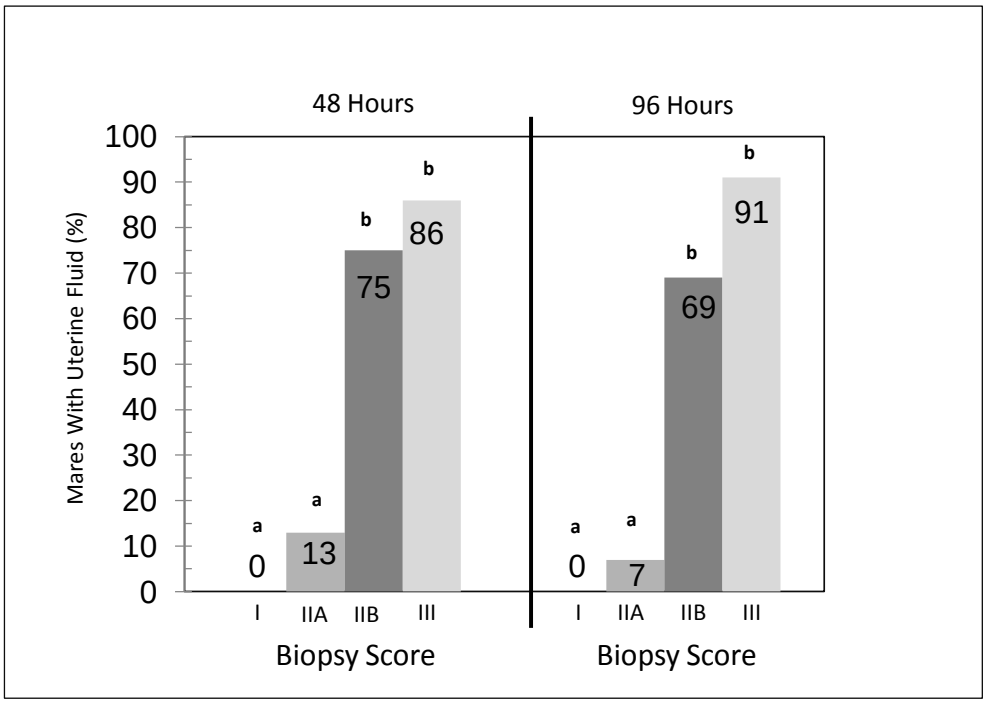
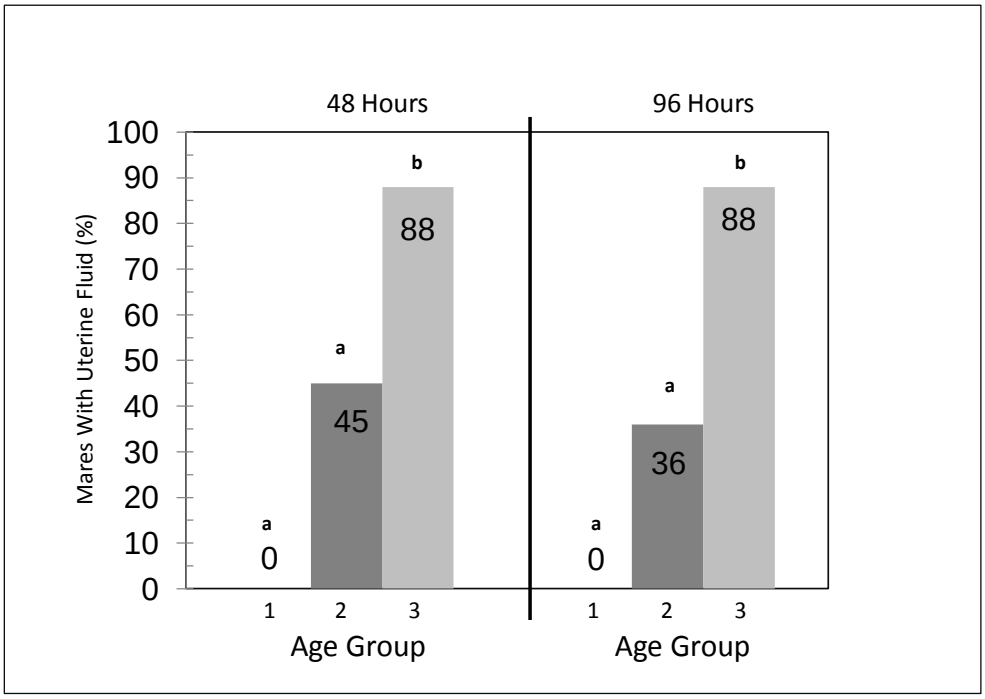


Fig. 2.2: Age group and intrauterine fluid in mares 48 and 96 hours after insemination. Columns without a common superscript differed ($P < 0.05$).



2.4.2 Experiment 2

Of the mares examined for susceptibility to PBIE, in the first season 3 mares were classified as resistant, 7 as intermediate, and 4 as susceptible. In the second season, 1 was resistant, 10 were intermediate, and 3 were susceptible (Table 2.1). Thirty-six percent (5 of 14) changed status with regards to susceptibility during subsequent seasons. Three mares (21%) changed to a more severe classification (intermediate to susceptible, or resistant to intermediate), whereas 2 mares (14%) changed to a less severe classification (susceptible to intermediate Table 2.1).

Table 2.1 Screening for persistent breeding induced endometritis (PBIE) in mares over subsequent seasons. Changes in classification status are marked with an asterisk (*) by the mare's identification number.

	Season 1				Season 2			
Mare	Biopsy	Cytology	Fluid	**Class**	Biopsy	Cytology	Fluid	**Class**
1	III	+	+	**S**	III	+	+	**S**
2*	IIB	+	+	**S**	IIB	-	-	**I**
3	I	+	-	**I**	IIA	+	-	**I**
4	I	+	-	**I**	I	+	-	**I**
5	IIB	-	+	**I**	IIB	-	-	**I**
6*	IIB	+	+	**S**	IIB	+	-	**I**
7	IIA	-	-	**R**	IIA	-	-	**R**
8	III	+	+	**S**	III	+	+	**S**
9*	IIB	-	-	**I**	III	+	+	**S**
10	IIB	+	-	**I**	III	+	-	**I**
11	IIA	+	-	**I**	IIA	+	-	**I**
12*	IIA	-	-	**R**	IIA	+	-	**I**
13*	IIA	-	-	**R**	IIB	-	-	**I**
14	IIA	+	+	**I**	IIA	+	+	**I**

2.5 Discussion

Based on this study, we inferred that a uterine biopsy score can be useful for most mares in predicting PBIE and intrauterine fluid retention as a response to insemination, although some mares may fall into an intermediate category. The present data supported findings from previous studies, as there was no difference in fluid retention between grades I and IIA or IIB and III (Troedsson *et al.* 1993a). The observation that groups IIB and III had increased incidences of fluid retention when compared to I and IIA further bolstered the classification criteria for susceptibility to PBIE, in that the line between potentially resistant and potentially susceptible can be drawn between biopsy scores IIA

and IIB. We concluded from the data that age was associated with fluid retention.

The foaling histories of the mares were unknown; therefore parity could not be included as a factor in the analysis of the data. Age combined with parity has been associated with endometrial degeneration, however it is difficult to separate the two to study the effect of parity alone on endometrial quality, as with increased parity comes increased age. Ricketts and Alonso (1991) investigated the relationship of chronic degenerative endometrial disease with age and parity, and found that maiden mares of increased age exhibited endometrial changes, indicating that age alone will be a causative factor in the development of endometrosis. In addition, these authors found that mares with increased parity had a higher than average incidence of poor endometrial quality, whereas mares with fewer foals had endometria with better than average quality. However, they also stated that the differences were too small to make any conclusions in a practical setting (Ricketts & Alonso 1991).

Another group of researchers used sub-fertile mares to investigate endometrial angiopathies as related to age and parity, and reported that mares of increased age and parity had a higher percentage of severe angiopathies, but evaluating the effect of parity alone was not possible (Gruninger *et al.* 1998). The study evaluated age as a sole factor when studying angiosis in maiden mares, and found that older maiden mares had a higher incidence of angiosclerotic changes than younger maiden mares. The authors then compared the occurrence of angiosis with that of endometrosis. Mares with angiosis also had a higher, more severe incidence of endometrosis, suggesting that had the authors looked at endometrial quality, they would have had similar results in regards to age and parity (Gruninger *et al.* 1998). Unfortunately, the conditions of the present study did not

permit a comparison of parity to endometrial quality and fluid retention, and parity cannot be excluded as a factor. However, evidence suggests that age alone is a factor in the progression of degenerative endometrial changes.

A mechanism for the association between uterine pathology (biopsy scores IIB and III) and the inability to clear fluid produced from an acute inflammatory response was not evaluated in this study. Intrauterine fluid is highly correlated with the presence of neutrophils which migrate through vessel walls to the uterine lumen during inflammation (Burleson *et al.* 2010). It was previously suggested that damage to blood vessels may lead to decreased circulation and lymphatic drainage, thereby increasing fluid retention and decreasing fertility (Gruninger *et al.* 1998). Doppler ultrasonography detected differences in uterine blood perfusion in mares with varying degrees of endometrial degenerative changes, further illustrating the importance of adequate blood flow and drainage for uterine function (Stolla & Bollwein 1997, Esteller-Vico *et al.* 2010). Repeated inflammation may also be associated with uterine fluid retention. Algamdi et al. (Alghamdi *et al.* 2005b) found an increase in mRNA expression of inducible nitric oxide synthase in endometrial inflammatory cells with a resulting increase of luminal nitric oxide production in susceptible mares after breeding, which may impair myometrial contractions, leading to decreased clearance through the lymphatic ducts and cervix. Another possible association between poor endometrial biopsy score and delayed uterine clearance is that prolonged inflammation can have an accelerating effect on the decline of the uterine health; it was found that repeated bouts of acute endometritis can lead to fibrotic changes (Hoffmann *et al.* 2009).

This study evaluated the clinical uterine inflammatory response to breeding.

Artificial insemination and (especially) natural cover are likely to introduce bacteria into the uterus unintentionally; therefore it is not possible to distinguish the inflammatory response to bacteria or sperm (Troedsson *et al.* 1995a). In any case, a resistant mare should be capable of clearing inflammation, sperm, and bacteria from the uterus within 48 h after breeding (Katila 1995). Although it is difficult to differentiate the inflammatory response to breeding with regards to bacteria and seminal exposure, we ensured that mares were free of ongoing bacterial infections prior to breeding. Those testing positive were removed from the experiment, treated, and returned to the experiment once a negative culture/cytology was obtained, therefore none of the mares tested positive initially at the start of the experiment.

Based on the results, we inferred that mares may change susceptibility to PBIE over subsequent breeding seasons. This change may be a gradual process, since none of the mares changed from resistant to susceptible, or from susceptible to resistant in subsequent seasons. Some mares improved their susceptibility status, suggesting that progression of PBIE is not unidirectional, but may develop over time with fluctuating degrees of resistance from one year to the next. The mares with improved resistance from the first to second year had unchanged biopsy scores for both seasons; their status improved because they did not have intrauterine inflammation (fluid, positive cytology, or both) in the second breeding season. Conversely, 2 of the mares worsened in status, due to the presence of uterine fluid and positive cytology. In addition, one resistant mare changed her status from the first to second year because her biopsy grade worsened from IIA to IIB, and she was therefore screened as a susceptible mare. She did not retain fluid or have positive cytology at 96 hours, and was classified as intermediate under the

conditions of this study. Two mares in Experiment 1 were free of fluid 48 hours after insemination, but did have intrauterine fluid 96 hours after breeding. It is unknown if these mares had developed bacterial infections at 48 hours (as they were not cultured at this time point in Experiment 1), but it is possible that they developed an infection after breeding, thereby causing fluid retention at 96 hours. All mares included in both experiments were free of bacterial infections and inflammation prior to breeding. Two of the grade I mares in Experiment 2 (mares number 3 and 4) did not have fluid retention 48 hours after breeding, but did have positive cytology, indicating the presence of inflammation. Because of the positive cytology, they were both considered intermediate mares. Had we evaluated the presence of neutrophils in the two mares in Experiment 1 at 48 hours, we may have seen similar findings. When taking into account the presence of neutrophils in addition to fluid retention, fewer horses may be considered resistant than when fluid retention is the sole factor as a marker of inflammation (none of the mares in experiment one with a grade I biopsy retained fluid 48 hours after insemination).

Three mares screened as susceptible in Experiment 2 were negative for cytology and fluid 96 hours after insemination. These mares failed to meet all of the criteria to be classified as resistant or susceptible for this study, and therefore were considered intermediate. However, it is unknown if these mares had inflammation at 48 hours, as potentially susceptible mares were only evaluated for inflammatory cells 96 hours after insemination. Any mare (regardless of biopsy score) capable of clearing fluid, inflammatory cells, and bacteria within 48 hours of breeding could be considered resistant to PBIE. However, using our strict criteria for ascertaining susceptibility to PBIE where biopsy grade may pre-classify a mare as susceptible or resistant, yet if

uterine inflammatory response after breeding fails to confirm this classification, mares would go into the intermediate category.

Although no statistical analyses were performed on the limited number of observations in Experiment 2, we inferred that mares were unlikely to improve their status because of biopsy score, as none of them improved their biopsy score. This finding was consistent with the association between age and biopsy score observed in this and other studies (Carnevale & Ginther 1992, Gruninger *et al.* 1998). To better ascertain the timeline for a mare's progression towards susceptibility to PBIE, a multi-year study would be appropriate, following mares until they reached susceptibility.

2.6 Conclusion

In conclusion, uterine fluid retention was associated with the severity of endometrial histopathology; combining biopsy score with ability to clear inflammation yielded a convincing set of criteria for identifying problem mares. In addition, although mares can be classified as susceptible or resistant to PBIE, many mares were classified in an intermediate group. Finally, although it was evident that mares will likely increase in susceptibility to PBIE with age and decreased endometrial quality, it was noteworthy that some mares improved their resistance from one season to the next.

2.7 Acknowledgments

This work was supported by funds from the Gluck Fellowship in the Department of Veterinary Science and the Janet H. Koller Endowment for Equine Research. The authors thank Mr. Lynn Ennis, Mr. Kevin Gallagher, and the University of Kentucky's

Maine Chance Farm for the management and care of the horses, the University of Kentucky Veterinary Diagnostic Laboratory for the use of their equipment and sample processing, and Dr. Gabriel Davolli and Ms. Pauline Peugnet for their technical assistance.

CHAPTER 3

Endometrial inflammatory markers of the early immune response in mares susceptible or resistant to persistent breeding induced endometritis (PBIE).

Woodward, E.M.[1], Christoffersen, M.[2], Campos, J.[1], Betancourt, A.[1], Horohov, D.[1], Scoggin, K.E.[1], Squires, E.[1], Troedsson, M.H.T[1].

[1]The Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, 40546-0099, USA; [2]Department of Large Animal Sciences, Veterinary Reproduction and Obstetrics, University of Copenhagen, Dyrlaegevej 68, DK-1870 Frederiksberg C, Denmark

*Submitted to Reproduction

3.1 Summary

Transient uterine inflammation after breeding is a physiological process necessary for clearance of bacteria and excess spermatozoa. In a subpopulation of mares, the inflammation fails to resolve in a timely fashion, resulting in a uterine environment detrimental to the conceptus. The objective of this study was to describe the inflammatory response in mares susceptible or resistant to persistent breeding induced endometritis (PBIE) during the first 24 hours after induction of uterine inflammation. Twelve mares were classified as susceptible (n = 6) or resistant (n = 6) to PBIE based on clinical response to insemination with dead spermatozoa and evaluation of endometrial quality. Mares were inseminated over 5 estrous cycles and endometrial biopsies were collected at one time point per cycle in randomized order before (0), and 2, 6, 12, and 24

hours after insemination. qPCR analysis for IL1β, IL6, IL8, IFNγ, TNFα, IL10, and IL1RA was performed using β-actin as a reference gene. Relative quantification values reported fold changes in mRNA expression from baseline (0 hours). A general pattern of expression post insemination was observed in both groups of mares. Cytokine mRNA increased at 2 hours, peaked at 2 to 6 hours, and then decreased. Differences were detected between groups of mares 6 hours after challenge; resistant mares had higher mRNA expression of IL6, IL1RA, and IL10 than susceptible mares. These findings describe an inherent difference in the initial immune response to insemination, and may help explain the transient nature of inflammation in resistant mares, whereas susceptible mares develop a persistent inflammation.

3.2 Introduction

A transient uterine inflammation in response to breeding is a normal physiological reaction that must be cleared within 5 days after ovulation, at which time the conceptus migrates into the uterus. Persistent inflammation beyond 5 days can be harmful to the conceptus and interfere with pregnancy (Oguri & Tsutsumi 1972, Betteridge *et al.* 1982, Troedsson *et al.* 1995b, Troedsson 1999). Uterine fluid retention 24-36 hours after breeding has been estimated to affect 10-15% of broodmares (Zent *et al.* 1998). While susceptibility to PBIE has been attributed in part to dysfunctional myometrial contractility (Troedsson & Liu 1991, LeBlanc *et al.* 1994), the underlying inflammatory mechanisms responsible remain poorly understood.

The inflammatory process is initiated by cell signaling molecules, followed by a series of signaling cascades that lead to the resolution of inflammation through the

recruitment of polymorphonuclear neutrophils (PMNs) and other inflammatory cells to the site of inflammation (Parham 2005). Understanding the mechanisms during the initial cell signaling period is important in describing subsequent inflammation. Cytokine mRNA expression during the uterine inflammation has been studied in the mare. Fumuso *et al*. investigated endometrial cytokine mRNA expression 24 hours after artificial insemination with semen in susceptible and resistant mares. The group detected an increase in pro-inflammatory cytokines interleukin (IL)1β, IL6, and tumor necrosis factor (TNF)α in all mares after insemination, and found that mares susceptible to PBIE had higher basal levels of mRNA expression of the cytokines when compared to resistant mares (Fumuso *et al.* 2003b). The same group subsequently found increased mRNA expression of the neutrophil chemoattractant IL8 and lower expression of the inflammatory modulating cytokine IL10 in susceptible mares compared to resistant mares 24 hours after insemination (Fumuso *et al.* 2006, Fumuso *et al.* 2007). However, in contrast to Fumuso *et al.*, other authors were unable to find a significant 24 hour IL8 response to insemination in reproductively normal pony mares (Nash *et al.* 2008, Nash *et al.* 2010). Endometrial mRNA expression of TNFα, IL1β, IL8, and IL10 were all found to be upregulated in response to a diestrus intrauterine inoculation of a high dose of *Escherichia coli* as early as 3 hours after challenge in normal mares (Christoffersen *et al.* 2010). In addition, uterine clearance begins as early as 30 minutes after insemination with radiocolloid (LeBlanc *et al.* 1994), and myoelectrical patterns in susceptible and resistant mares differ as early as 6 hours after bacterial inoculation into the uterus (Troedsson *et al.* 1993b). Given these findings, it is possible that the critical timeframe for cytokine mRNA expression as it relates to susceptibility to PBIE occurs much earlier than the 24 hours

time point previously investigated. Knowledge of the time frame of events and inflammatory signaling cascades occurring after breeding is critical for an understanding of the development of PBIE. Therefore, the objective of this study was to profile pro- and anti-inflammatory cytokine mRNA expression patterns in the equine endometrium at several time points within the first 24 hours after challenge with dead spermatozoa. We hypothesized that (1) there is a change in the endometrial inflammatory cytokine mRNA expression in mares at varying time points within 24 hours after insemination, and (2) that mares susceptible to PBIE have a different profile of cytokine expression than mares resistant to PBIE within the first 24 hours of breeding.

3.3 Materials and Methods

3.3.1 Animals

Ninety-two mares (*Equus caballus*) of mixed breeds, aged (2 to 27 years), and foaling history were kept on pasture supplemented with grain and hay, and were provided with water and salt *ad-libitum*. Three stallions were maintained under similar housing conditions to that of the mares. The experiment was approved by the University of Kentucky's IACUC (protocol numbers 2009-0455 and 2009-0602).

3.3.2 Preparation of sperm for insemination

Semen was regularly collected from the stallions and used as needed throughout the experiment. Freeze-killed spermatozoa were prepared by removing the seminal plasma from ejaculates using centrifugation (2000 × g for 10 minutes), resuspending the spermatozoa pellet in milk based extender (EquiPro™, Minitube, Verona, WI, USA) in

aliquots of 1×10^9 spermatozoa in 30 mL of extender, followed by at least 2 freeze (−20°C)/thaw cycles of the aliquots to kill the spermatozoa.

3.3.3 General experimental procedure for the selection of mares

Mares were examined for susceptibility to PBIE based on endometrial histology and the results of a spermatozoa challenge. Endometrial biopsies (approximately 300 mg of tissue) were obtained during diestrus using an alligator jaw biopsy instrument, fixed in 10% formalin, sectioned at 5 μm and stained with hematoxylin and eosin. Each biopsy was examined for periglandular fibrosis, inflammatory cells, glandular distribution and lymphatic lacunae, then graded according to Kenney and Doig (Kenney & Doig 1986). Mares with scores of I or IIA were considered potentially resistant to PBIE (n = 27), while those with scores of IIB or III were potentially susceptible to PBIE (n = 65) (Troedsson *et al.* 1993a).

After selection as potentially resistant or susceptible to PBIE, the mares' reproductive tracts were observed regularly for signs of estrus and pending ovulation using transrectal ultrasonography. Estrus was defined by the presence of uterine edema, a relaxed cervix, and at least one follicle 35 mm or larger in diameter in the absence of a *corpus luteum*. Once estrus was detected, mares were evaluated for the presence of intrauterine inflammatory cells using a cytobrush (Minitube) or low volume lavage to obtain samples, and for intrauterine bacteria using a double-guarded swab (Minitube) to obtain samples, and then were subsequently inseminated with freeze-killed spermatozoa (prepared as above). For the cytological evaluations, a sample positive for inflammation was defined as more than 2 neutrophils per 5 fields at ×400 magnification. Swab samples

were cultured on blood agar, then incubated at 37°C for 24 hours and evaluated for growth. Mares were challenged with an intrauterine inoculation of 1×10^9 freeze-killed stallion spermatozoa in 30 mL of milk based semen extender (Minitube). Only mares with negative cytology and no bacterial growth prior to insemination were considered for the experiment.

Potentially susceptible mares were evaluated 96 hours after insemination and were confirmed as a susceptible mare if they had 1) positive cytology, and 2) uterine fluid retention. Potentially resistant mares were assessed 48 hours after breeding, and those with 1) negative cytology and culture in addition to 2) no uterine fluid retention were confirmed as resistant mares (Troedsson & Liu 1991, Alghamdi *et al.* 2005b). All mares not meeting the criteria for classification as susceptible or resistant were considered as intermediate and excluded from the study. From the herd of 92 mares, a total of 6 susceptible and 6 resistant mares were identified and used in this study.

3.3.4 *Treatments*

Over 5 subsequent cycles, estrus was induced through im administration of prostaglandin F2α, (Lutalyse® 7.5 mg, Pfizer, New York, NY, USA). Once estrus was detected, mares were evaluated for cytological and bacterial findings (as described above), and then subsequently inseminated with 1×10^9 dead spermatozoa (as described above). Only estrous cycles with negative cytology and culture were used for the experiment; if mares were positive for cytology or culture at the time of insemination, they were treated and insemination attempted again during a subsequent cycle.

Uterine biopsies were collected at one time point per cycle in randomized order. Time points were: 0 (prior to insemination), 2, 6, 12, and 24 hours after insemination. The obtained tissue was either snap frozen in liquid nitrogen and stored at −80°C until further processing, or stored in RNAlater® (Applied Biosystems, Carlsbad, CA, USA) overnight at 4°C, then moved to −20°C for storage until further processing.

3.3.5 qPCR analysis

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated with a commercially available kit (DNA-free™, Applied Biosystems), then analyzed for quality and quantity using a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The ranges for the 260/280 and 260/230 ratios were 1.92-2.2 and 1.89-2.4, respectively. 1.5 µg of RNA in 41.5 µL ddH_2O was reverse transcribed in a reaction using AMV Reverse Transcriptase (0.5 µL;10 U/µL), 5×RT Buffer (16 µL), RNAsin® (1 µL; 40 U/µL), MgCl (16 µL; 25 mM), dNTP (4 µL;10 mM), and Oligo(dT) Primer (1 µL; 500 µg/mL) (all reagents from Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 minutes, then 95°C for 5 minutes. cDNA was diluted 1:1 with ddH_2O, and qPCR for each sample was performed using 4.5 µL of cDNA, 5 µL of Sensimix™ II (Bioline, Tauton, MA, USA), and 0.5 µL of a custom dual hydrolysis primer/probe set (Applied Biosystems) (Table 3.1). Reactions were performed in duplicate, and using the 7900HT Fast Real-Time PCR System (Applied Biosystems), were incubated at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. PCR efficiencies were calculated using

LinRegPCR (version 7.0). β-actin was used as the reference gene, as it was previously determined to be the most stably expressed gene out a panel of 4 reference genes (18 S RNA, β-glucoronidase, glyceraldehyde 3-phosphate dehydrogenase, β-actin) (Christoffersen *et al.* 2010). Results are expressed as mean relative quantification values (RQ) which were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), with the calibrator as the mean cycle threshold (ΔC_T) value of the 0 hour collections from the resistant mares.

Data were analyzed using SigmaStat© (Systat Software, Inc.) with two-way repeated measures ANOVA tests. Data were log10 or square root transformed for normal distribution, and outliers were defined as ± 2 SD from the mean and were removed from statistical analyses (5 of 406 data points removed). Missing data points were automatically calculated by the software using a general linear model **(**Sigma Stat User Guide, p. 379). Comparisons were made between groups at each time point, and between time points within groups. Significance was set to $P < 0.05$.

3.4 Results

IL1β mRNA expression was increased from baseline levels at 2 hours and 6 hours for both groups of mares ($P \leq 0.01$). No differences were observed between susceptible and resistant mares at any time point (Figure 3.1).

IL6 mRNA expression was increased from baseline levels at all time points in both groups of mares ($P \leq 0.047$). In addition, susceptible mares had a higher level of expression at 2 hours when compared to 24 hours ($P = 0.033$) and resistant mares had an

increase at 6 hours when compared to 12 and 24 hours ($P \leq 0.002$). Resistant mares had a higher level of expression at 6 hours when compared to susceptible mares ($P = 0.01$) (Figure 3.2).

IL8 mRNA expression in resistant mares was increased at 2 and 6 hours from baseline ($P < 0.04$), followed by a decrease from 6 to 12 hours ($P = 0.042$). In susceptible mares, there was a significant rise in expression from baseline to 2 hours ($P = 0.005$). There were no differences in expression between susceptible and resistant mares (Figure 3.3).

Interferon gamma (IFNγ) mRNA expression in resistant mares was increased from baseline at 2 hours and 6 hours ($P \leq 0.001$) after insemination. Susceptible mares had increased expression at 6 hours compared to baseline, 12, and 24 hours ($P \leq 0.046$). There were no differences between susceptible and resistant mares; however, mRNA expression patterns of IFNγ differed between the two groups shortly after insemination. Although both groups exhibited a response, the resistant mares had increased expression at 2 hours, whereas the susceptible group did not increase until 6 hours after insemination (Figure 3.4).

TNFα mRNA expression in susceptible mares did not differ at any time point. In resistant mares, there was an increase in expression at 2 hours from baseline, 12, and 24 hours ($P \leq 0.032$). Expression then decreased at 12 hours from 2 and 6 hours ($P \leq 0.034$), and at 24 hours was lower than baseline ($P = 0.046$). There were no differences between groups (Figure 3.5).

IL10 mRNA expression in resistant mares was up-regulated at 6 hours compared to all time points ($P \leq 0.001$), and at 2 hours compared to baseline and 24 hours ($P \leq$

0.045). Susceptible mares showed an increase at 6 hours compared to baseline and 24 hours ($P \leq 0.027$). Resistant mares had higher expression than susceptible mares at 6 hours ($P = 0.021$) (Figure 3.6).

IL1RA mRNA expression peaked at 6 hours in resistant mares ($P < 0.001$), with a marked increase from 2 to 6 hours ($P < 0.001$) and a decrease from 6 to 12 hours, ($P < 0.001$). Expression returned to baseline levels by 24 hours. Susceptible mares had increased expression from baseline at all time points ($P \leq 0.017$), with the highest levels observed at 6 and 12 hours. Resistant mares had higher mRNA expression at 6 hours than susceptible mares ($P = 0.013$), and susceptible mares had higher expression at 12 hours than resistant mares ($P = 0.017$) (Figure 3.7).

The general mRNA expression patterns for the pro-inflammatory cytokines IL1β, IL8, IFNγ, and TNFα, were similar in both groups of mares, with an initial increase at 2 and 6 hours, followed by a decrease towards baseline levels. Although there were no differences between susceptible and resistant mares at any time point for these cytokines, there were subtle variations in the rise towards the peak and the return to baseline level expression. All mares returned to baseline levels of mRNA expression of IL1βand IL8 by 12 hours. However, resistant mares had a significant decrease in expression from 6 to 12 hours, while the susceptible mares had a less rapid decline in expression to pre-insemination levels compared to the resistant mares.

There were distinct differences in the patterns of expression between resistant and susceptible mares for IL6 and the inflammatory modulating cytokines IL1RA and IL10. mRNA expression for all three cytokines was decreased in the susceptible mares at 6 hours when compared to the resistant mares, and susceptible mares had a higher

expression of IL1RA at 12 hours when compared to the resistant mares. An outline connecting the mean expression at each time point for the three cytokines shows that the resistant mares have an expression profile with a sharp peak at 6 hours, while the lines for the susceptible mares resemble more of a hump, with slower increases to and decreases from the peak (Figures 3.2, 3.6, 3.7).

Table 3.1: Primer probe set sequences for the detection of equine mRNA. Primer probe sets were designed using Assays-By-Design (Applied Biosystems, Carlsbad, CA)

Cytokine	**Forward primer sequence**	**Reverse primer sequence**	**Probe sequence**
Eqβ-actin	CGCAAAGCAATAAGTGAACTCATCAA	GCCTCGAAACGGATTCTGACT	CTTCCTCAGGTTAGCTTTG
EqIL1	CCCAAACTCTCCAAGATTCTCACAT	CAGAGGTTTGAGTTCTTCTTCTAGACA	ATGCCCAAGAAGGCCAC
EqIL6	TGACTGTAGCGGATGCCTTTG	GCCCTGCAGATTTCCTTTCCAT	CTGGCCCGAAGAAC
EqIL8	CCTGGAGTCCCTGAGCAA	CATCTTCCGCGTGTTTTGGAT	TCTCCACCTGCAGTGCC
EqINFγ	AGCAGCACCAGCAAGCT	TTTGCGCTGGACCTTCAGA	ATTGAGATTCCGGTAAATG
EqTNFα	TTACCGAATGCCTTCCAGTCAAT	GGGCTACAGGCTTGTCACTT	CCAGACACTCAGATCAT
EqIL10	ATGCCCCAGGCTGAGAAC	CGGAGGGTCTTCAGCTTTTCC	CCAGACATCAAGGAGCACG
IL1RA	AGTTGCTGGATACTTGCAAGAATCA	GAGTCCCAGGAATAGAGCATCAG	CATCTATCTTCTCTTGTAATTTA

Figures 3.1-3.7: Mean endometrial cytokine mRNA expression before (0) and 2, 6, 12, 24 hours after insemination with killed spermatozoa in susceptible (S; dark bars) and resistant (R; light bars) mares. RQ values describe fold changes in each mare compared to the ΔC_T value of resistant mares at 0 hours. Data presented in graphs are the raw RQ values, although data was log10 or square root transformed for statistical analysis. Error bars report standard error of the mean. Comparisons were made within groups (S or R) at each time point and different letters over the bars (capital letters for susceptible; lowercase for resistant) within each group represent significant differences. Comparisons were also made between groups at each time point, and significant differences are represented by an asterisk (*). Significance set to $P < 0.05$.

Figure 3.1

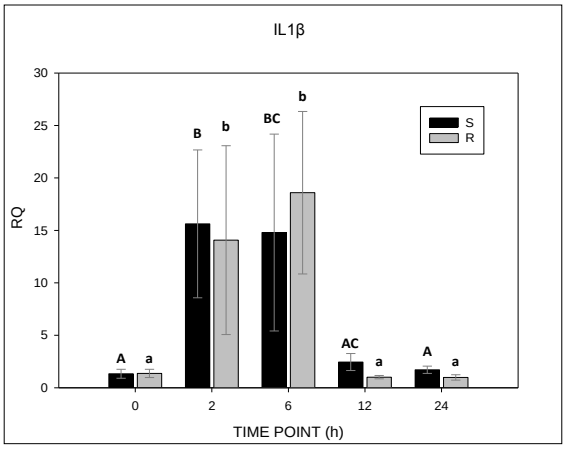


Figure 3.2

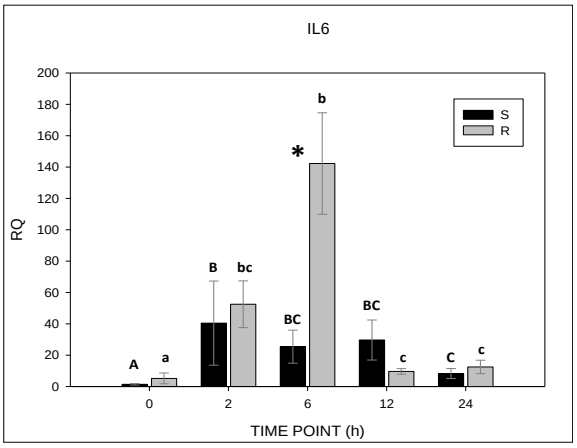


Figure 3.3

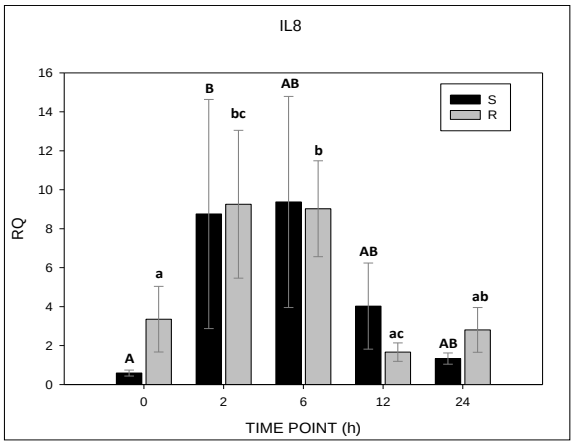


Figure 3.4

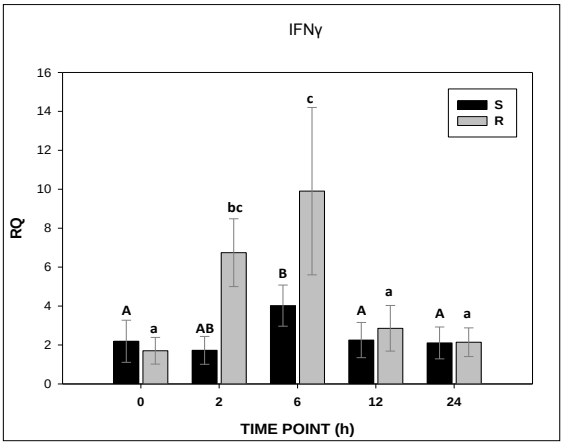


Figure 3.5

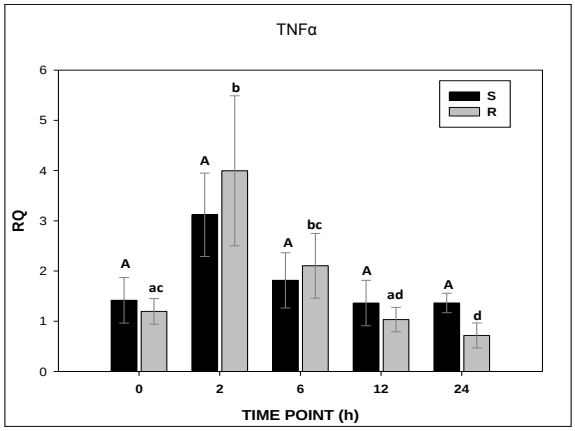


Figure 3.6

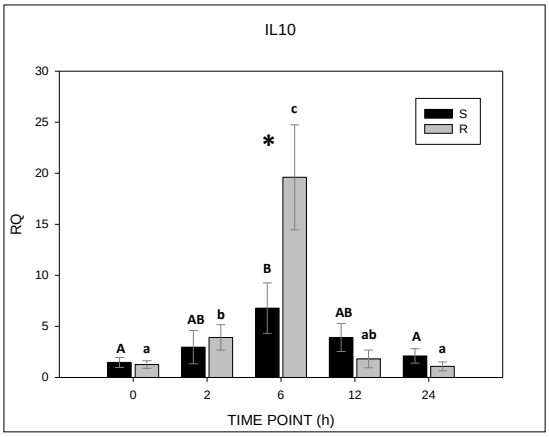
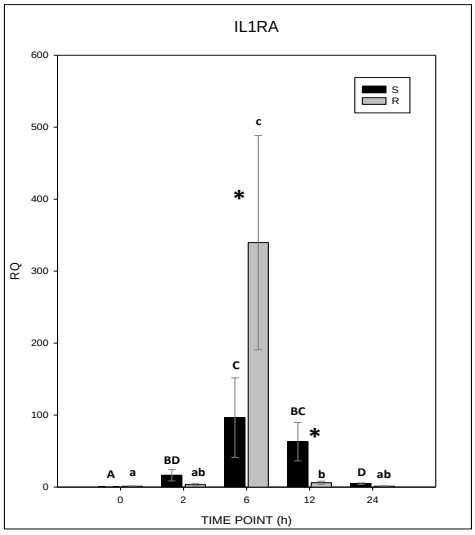


Figure 3.7



3.5 Discussion

Pro-inflammatory cytokines initiate inflammation, and once produced, a cascade of inflammatory events occurs (Kuby 1992, Parham 2005). A timely response to pathogens is important for an effective resolution of inflammation and preservation of tissue, and IFNγ is important in the initiation of inflammation (Schroder *et al.* 2004). The delayed rise in IFNγ mRNA in the susceptible mares observed in this study may reflect a delayed inflammatory response to spermatozoa compared to resistant mares. Furthermore, the differences observed in the mRNA patterns of expression for the pro-inflammatory cytokines suggest that susceptible mares have a slower return to baseline levels, possibly contributing to the prolonged clinical signs observed in the susceptible mares (Zent *et al.* 1998).

As with the other pro-inflammatory cytokines, mRNA expression for IL6 increased in response to challenge in both groups. IL6 was the only pro-inflammatory cytokine with a significant difference between the two groups, with resistant mares having increased mRNA expression compared to susceptible mares at 6 hours, which is similar to the findings of the inflammatory modulating cytokines IL10 and IL1RA. While IL6 is considered a pro-inflammatory cytokine, it has protective roles in early acute inflammation and shock through the modulation of other pro-inflammatory cytokines (Barton & Jackson 1993, Xing *et al.* 1998, Diao & Kohanawa 2005), and the induction of inflammatory modulating cytokines, such as IL1RA (Tilg *et al.* 1994). In addition to the resolution of acute inflammation, IL6 and its receptor has been suggested to have implications in the transition from innate to acquired immunity and the persistence of chronic inflammation (Kishimoto *et al.* 1995, Atreya *et al.* 2000, Jones 2005). It is

possible that the increase of IL6 mRNA 6 hours after insemination in resistant mares compared to susceptible mares may reflect initial inflammatory modulating roles of IL6 (similar to those of IL10 and IL1RA) during this time (Tilg *et al.* 1994). In mares, treatment with the anti-inflammatory dexamethasone in susceptible mares has been demonstrated to increase endometrial IL6 mRNA expression 3 hours after inoculation with *E. coli* when compared to susceptible mares inoculated without dexamethasone treatment (Christoffersen, *et al.* 2011). The same group observed an increase in mRNA expression of the immune modulating cytokine IL10 and a decrease in the pro-inflammatory cytokine IL1β at 3 hours after dexamethasone treatment. These findings further support that IL6 may be initially involved with an immune modulating response to challenge, as the initial response of IL6 to dexamethasone is similar to that of the immunomodulator IL10, and opposite to that of the pro-inflammatory IL1β. In addition, the elevated levels of IL6 mRNA observed in untreated susceptible mares inoculated with *E. coli* at 72 hours is similar to the response of the IL1β, and opposite to IL10 (Christoffersen, *et al*. 2011), which may reflect the pro-inflammatory actions of IL6 associated with inflammation more persistent in nature.

There is a delicate balance between the pro and anti-inflammatory response. Pro-inflammatory cytokines act as signaling molecules to initiate and increase the response, while anti-inflammatory cytokines modulate the pro-inflammatory cytokines (Parham 2005, Hackett *et al.* 2008). IL10 is essential for the proper function of the immune response, and deficiencies in IL10 can lead to tissue damage (Cyktor & Turner 2011). In addition, the IL1 system is involved with both pathologic states as well as normal reproductive function (Takehara *et al.* 1994, Van der Hoek *et al.* 1998, Martoriati *et al.*

2003, Gerard *et al.* 2004). IL1RA mediates IL1, which if overactive, can also damage tissue (Gerard *et al.* 2004). In this study, susceptible mares had a less defined immune modulating response than resistant mares, suggesting that these mares were less able to respond to and initiate the modulation of inflammation.

These data support previous research that illustrated a local immune response in response to intrauterine challenge with dead spermatozoa (Fumuso *et al.* 2007), and that susceptible mares are less capable of clearing the uterine inflammation than resistant mares (Kenney & Doig 1986, Fumuso *et al.* 2006). However, the results conflict with that of other studies as to which cytokines differ and at which time points (Fumuso *et al.* 2003b, Fumuso *et al.* 2006). A possible explanation for discrepancies between studies is the criteria by which mares are selected as resistant versus susceptible to PBIE. It is important to determine that mares are free of inflammation prior to breeding (via uterine culture and cytology), because chronic inflammation may change cytokine mRNA profiles at the baseline level, therefore altering results if data are to be analyzed using a relative quantification method, as may have been the case in the later studies. A critical factor for classification of mares is to ensure that the criteria for susceptibility and resistance are stringent enough to represent the truly resistant or susceptible mare, since many mares fall into an intermediate category. The model used in this experiment utilizes such strict criteria and therefore is appropriate for the study of PBIE. In addition, these data demonstrate that the time point of 24 hours may be too late to see a response with some cytokines in normal mares, which could explain inconsistencies with this experiment and previously published works (Fumuso *et al.* 2006, Nash *et al.* 2010). For example, if normal mares are meant to resolve inflammation within 48 hours, it is

plausible that at 24 hours after challenge, neutrophils are no longer being actively recruited, and although neutrophils are likely to still be present in the uterus (Nash *et al.* 2010), IL8 may not be produced at levels above baseline during this time.

The findings that susceptible mares have an altered initial inflammatory response on a gene expression level support other data describing differences in the ability to clear inflammation between resistant and susceptible mares after bacterial challenge (Troedsson & Liu 1991, Troedsson *et al.* 1993b, LeBlanc *et al.* 1994). Although the clinical signs differentiating resistant and susceptible mares are observed several days after insemination, to better define the pathogenesis of PBIE it is important to understand on a molecular level the timing of the uterine immune response of the resistant mare compared to the susceptible mare. These data suggest that around 6 hours after insemination is a critical time in developing susceptibility, as this time point was both the peak of expression observed for many of the cytokines, and the time point at which susceptible and resistant mares differed in their mRNA expression of IL6, IL10, and IL1RA. Furthermore, a failure to resolve breeding induced inflammation in a timely fashion may be due in part to a failure to mobilize inflammatory (both pro- and anti-inflammatory) cytokines during the early inflammatory period, which could contribute to a delayed resolution of inflammation in susceptible mares.

3.6 Conclusion

In conclusion, the results from this study suggest that 6 hours after insemination with spermatozoa is a critical time to set up the mechanism for uterine response and clearance. It is possible that the altered local cytokine response observed in susceptible

mares could lead to increased inflammation and inflammatory byproducts (such as nitric oxide), which in turn could lead to an impaired ability to clear inflammation, as the cytokine response to inflammation is the first step in recognition and removal of foreign material. With an increased understanding of the cytokine pathways shortly after breeding, research can continue in elucidating the mechanisms in later stages of inflammation, and eventually, treatment strategies can be improved upon.

3.7 Acknowledgments

The authors would like to thank Mr. Lynn Ennis, Mr. Kevin Gallagher, and the University of Kentucky's Maine Chance Farm for the management and care of the horses, the University of Kentucky Veterinary Diagnostic Laboratory for the use of their equipment and sample processing, and Dr. Gabriel Davolli and Ms. Pauline Peugnet for their technical assistance.

CHAPTER 4

The effect of immune modulation on endometrial cytokine expression in mares susceptible to persistent breeding induced endometritis.

Woodward, E.M.[1], Christoffersen, M. [2], Horohov, D. [1], Squires, E.L. [1], Troedsson, M.H.T. [1]

[1]The Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, 40546-0099, USA; [2]Department of Large Animal Sciences, Veterinary Reproduction and Obstetrics, University of Copenhagen, Dyrlaegevej 68, DK-1870 Frederiksberg C, Denmark

Will be submitted to Equine Veterinary Journal

4.1 Summary

Transient uterine inflammation after breeding is a physiological process necessary for clearance of bacteria and excess spermatozoa. In a subpopulation of mares, the inflammation fails to resolve in a timely fashion, resulting in a persistent breeding induced endometritis (PBIE) that is detrimental to the conceptus. Previous research has shown that 6 hours after breeding is a critical time in the alteration of the uterine innate immune system of mares susceptible to PBIE. The specific aim of this experiment was to evaluate the effects of immune modulation with dexamethasone and mycobacterial cell wall extract (MCWE) on endometrial mRNA expression of inflammatory genes in susceptible mares 6 hours after breeding. We hypothesized that these treatments will alter cytokine expression when compared to non-treated susceptible mares. Six mares were

classified as susceptible to PBIE based on their inflammatory response to insemination with dead spermatozoa (intrauterine fluid accumulation, uterine culture, and cytology). After selection for the study, mares were inseminated during 3 consecutive estrus cycles with 1×10^9 killed spermatozoa and one of the following treatments: 1) alone (control), or in combination with 2) dexamethasone (50 mg iv) at the time of insemination, or 3) with MCWE (Settle™, 1.5 mg iv) administered 24 hours prior to insemination. All mares received one treatment per cycle in randomized order, and each mare served as her own control. Endometrial biopsies were collected 6 hours after breeding, and qPCR analysis for IL1β, IL6, IL8, IFNγ, TNFα, TLR2, TLR4, IL10, and IL1RA was performed using β-actin as a reference gene. Relative quantification values reported fold changes in mRNA expression from the control. Data were analyzed using an ANOVA and significance was set at $P < 0.05$. Expression of IL1β mRNA was lower after treatment with dexamethasone ($P < 0.001$) and MCWE ($P = 0.046$) when compared to control. No differences were detected in the mRNA expression of IL1RA, IL6, TLR2, and TLR4 after any of the treatments. In conclusion, treatment with immune modulators alters mRNA expression of pro-inflammatory IL1 in the uterus after insemination, which may alter the course of uterine inflammation.

4.2 Introduction

Uterine inflammation in response to breeding is a normal physiological reaction that must be cleared well within 5 days after ovulation, at which time the conceptus migrates into the uterus (Oguri & Tsutsumi 1972, Betteridge *et al.* 1982, Troedsson 1999). Persistent breeding induced endometritis (PBIE) can interfere with pregnancy (Troedsson *et al.* 1995b) and is a major cause of infertility in mares. Mares have been

classified as susceptible to PBIE based on reproductive history, fluid retention after breeding (Brinsko *et al.* 2003), failure to clear a bacterial and sperm challenge (Troedsson *et al.* 1993a, Troedsson *et al.* 1993b, Alghamdi *et al.* 2005b, Woodward *et al.* 2011a, Christoffersen *et al.* 2012), and endometrial pathology (Troedsson *et al.* 1993a, Woodward *et al.* 2011b).

Cytokines are involved with the initiation of the inflammatory response (Kuby 1992, Parham 2005), and have recently been the focus of research on PBIE. In one study, an increase in mRNA levels of pro-inflammatory cytokines interleukin (IL)1, IL6, and tumor necrosis factor alpha (TNFα) was detected in all mares 24 hours after insemination, but susceptible mares had higher basal levels of the cytokines than resistant mares (Fumuso *et al.* 2003b). The same researchers subsequently found increased mRNA expression of IL8 and lower expression of the inflammatory modulating cytokine IL10 in susceptible mares compared to resistant mares 24 hours after insemination (Fumuso *et al.* 2006, Fumuso *et al.* 2007). However, other authors were unable to find a significant IL8 response to insemination in reproductively normal pony mares 24 hours after insemination (Nash *et al.* 2008, Nash *et al.* 2010).

In another study, the inflammatory cytokine response to spermatozoa at 2, 6, 12 and 24 hours after insemination was investigated (Woodward *et al.* 2011a). Susceptible mares had lower expression of the inflammatory modulating cytokines IL10 and IL1 receptor antagonist (IL1RA), and the pro-inflammatory cytokine IL6 (which can act as an inflammatory modulator in the initial stages of inflammation (Tilg *et al.* 1994) when compared to resistant mares 6 hours after insemination. This indicates that 6 hours after insemination is a critical time for the development PBIE, as inflammatory cytokine

mRNA expression differed between susceptible and resistant mares during this time (Woodward *et al.* 2011a).

Currently, there are a variety of treatment strategies available for correcting PBIE, including administration of ecobolics, uterine lavage, antibiotics, and mucolytics. These treatments are often used in combination, with variations in dose, frequency, duration of treatment, and administration route (Combs *et al.* 1996, Watson 2000, Bliss & Campbell 2002, Hurtgen 2006, Liu & Troedsson 2008, LeBlanc 2010). The use of immune modulators to treat problem mares has also been proposed as an effective treatment strategy. Pregnancy rates were improved in mares with a history of fluid retention after breeding when treated with 5 doses of predinisolone acetate (Dell' Aqua *et al.* 2006). Furthermore, oral administration of 200 mg of dexamethasone twice daily for 5 days before breeding improved pregnancy rates in sub-fertile mares (Morris & Eden 2008). When condensing treatments into one dose, Bucca *et al*. (2008) saw a reduction in clinical signs of PBIE with iv administration of dexamethasone at the time of breeding, and concluded that a single dose of 50 mg dexamethasone is both safe and effective for the treatment of PBIE in mares with at least 3 risk factors for the condition (Bucca *et al.* 2008).

Mycobacterial cell wall extract (MCWE) has been used to stimulate the immune response to treat cancer (Filion & Phillips 2001, Yuksel *et al.* 2011), respiratory disease, and as a vaccine adjuvant (Berinstein *et al.* 1991). Several studies investigated the effects of MCWE on reproductive function in mares, and found that MCWE decreased the number of mares with endometritis after challenge with *Streptococcus zooepidemicus* (Rogan *et al.* 2007), and that pregnancy rates were increased in mares bred during their

foal heat cycle (Fumuso *et al.* 2003a). In addition, MCWE was shown to decrease levels of pro-inflammatory cytokines in susceptible mares towards levels of resistant mares 24 hours after breeding (Fumuso *et al.* 2003b, Fumuso *et al.* 2007).

Although the efficacy of immune modulators for the treatment of PBIE has been investigated, data on the mechanism of action for these drugs is sparse. The specific aim of this experiment was to evaluate the effects of dexamethasone and MCWE on the expression of inflammatory cytokine mRNA in mares susceptible to PBIE at 6 hours after insemination, with a hypothesis that these treatments will alter cytokine expression when compared to non-treated susceptible mares.

4.3 Materials and Methods

4.3.1 Animals

Mares of mixed breeds, age (15-26 years), and breeding history were kept on pasture supplemented with grain and hay, and were provided with water and salt *ad-libitum*. Semen was provided by 3 stallions with similar housing conditions to that of the mares. The experiment was approved by the University of Kentucky's IACUC (protocol numbers 2009-0455 and 2009-0602).

4.3.2 Preparation of sperm for insemination

Freeze-killed spermatozoa were prepared by removing the seminal plasma from ejaculates using centrifugation, resuspending the spermatozoa pellet in milk based extender in aliquots of 1×10^9 spermatozoa in 30 mL of milk based semen extender (Equi-

Pro; Minitube, Verona, WI), followed by at least 2 freeze (−20°C)/thaw (room temperature) cycles of the aliquots.

4.3.3 General experimental procedure

Mares were examined for susceptibility to PBIE based on endometrial histology and the results of a spermatozoa challenge. Endometrial biopsies (approximately 300 mg of tissue) were acquired during diestrus using an alligator jaw biopsy instrument, fixed in 10% formalin, sectioned at 5 μm and stained with hematoxylin and eosin. Each biopsy was examined for periglandular fibrosis, inflammatory cells, glandular distribution and lymphatic lacunae, then graded according to Kenney and Doig (Kenney & Doig 1986). Mares with scores of IIB or III were considered as potentially susceptible to PBIE (Troedsson *et al.* 1993a). After selection as potentially susceptible to PBIE, the reproductive tracts of the mares were observed regularly using transrectal ultrasonography, and were inseminated with freeze-killed spermatozoa (prepared as described above) during estrus in the presence of uterine edema, a relaxed cervix, and at least 1 follicle 35 mm or larger in diameter in the absence of a *corpus luteum*. Prior to inseminations, mares were evaluated for the presence of intrauterine inflammatory cells using a cytobrush (Minitube) or low volume lavage to obtain samples, and for intrauterine bacteria using a double-guarded swab (Minitube) to obtain samples. Inflammation was defined as more than 2 neutrophils per 5 fields at ×400 magnification. Only mares with negative cytology and culture prior to insemination were considered for the experiment; if mares were positive for cytology or culture at the time of insemination, they were treated and insemination was attempted again during the following cycle.

Potentially susceptible mares were evaluated 96 hours after insemination and were confirmed as susceptible if they had 1) positive cytology with either positive or negative culture, and 2) uterine fluid retention (Troedsson & Liu 1991, Alghamdi *et al.* 2005b). All mares not meeting the criteria for classification as susceptible were excluded from the study. A total of 6 susceptible mares (age 15-24 years) were identified and used for the experiment.

4.3.4 Treatments

Estrus was induced through administration of prostaglandin, (Lutalyse®, 7.5mg, Pfizer) over 3 subsequent cycles. Once estrus was detected, mares were evaluated for endometrial cytological and bacterial findings. Mares with negative cytology and uterine culture results were administered with one of the following treatments: 1) insemination with freeze-killed spermatozoa, 2) 50 mg dexamethasone iv and insemination with freeze-killed spermatozoa, and 3) 1.5 mg MCWE (Settle™, Bioniche, 1.5 mg) iv when a follicle ≥ 30 mm and uterine edema was present, and insemination with freeze-killed spermatozoa 24 hours after treatment. All mares received each treatment in randomized order.

4.3.5 Sample collection: endometrial tissue

Endometrial biopsies were collected 6 hours after breeding, and stored in RNAlater® (Applied Biosystems, Carlsbad, CA) overnight at 4°C, then moved to −20°C for storage until further processing.

4.3.6 qPCR analysis

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA), precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated with a commercially available kit (DNA-free™, Applied Biosystems), then analyzed for quality and quantity using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). 1.5 µg of RNA in 41.5 µL ddH_2O was reverse transcribed in a reaction using AMV Reverse Transcriptase (0.5 µL;10 u/µL), 5×RT Buffer (16 µL), RNAsin (1 µL; 40 u/µL), MgCl (16 µL; 25 mM), dNTP (4 µL;10 mM), and Oligo(dT) Primer (1 µL; 500 µg/mL) (all reagents from Promega, Madison, WI). Samples were incubated at 42°C for 60 minutes, then 95°C for 5 minutes. cDNA was diluted 1:1 with ddH_2O, and qPCR for each sample was performed using 4.5 µL of cDNA, 5 µL of Sensimix™ II (Bioline, Tauton, MA), and 0.5 µL of custom a primer/probe set (Applied Biosystems) (Table 4.1). Reactions were performed in duplicate using the 7900HT Fast Real-Time PCR System (Applied Biosystems), and were incubated at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. PCR efficiencies were calculated using LinRegPCR (version 7.0). Results are expressed as mean relative quantification values (RQ) which were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), with the calibrator as the mean cycle threshold (ΔC_T) value of the control samples.

qPCR data were analyzed using SigmaStat© (Systat Software, Inc.) with a one way repeated measures ANOVA. Prior to analysis, data were log10 transformed for normal distribution when needed, and Kruskal-Wallis one way ANOVA on Ranks was

used for data that was not normally distributed after transformation. Outliers were defined as ± 2 SD from the mean and were removed from statistical analyses (4 of 123 total data points; 3.2%). Comparisons were made between treatments for the susceptible mares, and significance was set to $P < 0.05$.

Table 4.1: Primer probe set sequences for the detection of equine mRNA. Primer probe sets were designed using Assays-By-Design (Applied Biosystems) and TaqMan® Custom Gene Expression Assay for TLR2 and TLR4 (Applied Biosystems).

Cytokine	**Forward primer sequence**	**Reverse primer sequence**	**Probe sequence**
Eqβ-ACTIN	CGCAAAGCAATAAGTGAACTCATCAA	GCCTCGAAACGGATTCTGACT	CTTCCTCAGGTTAGCTTTG
EqIL1β	CCCAAACTCTCCAAGATTCTCACAT	CAGAGGTTTGAGTTCTTCTTCTAGACA	ATGCCCAAGAAGGCCAC
EqIL6	TGACTGTAGCGGATGCCTTTG	GCCCTGCAGATTTCCTTTCCAT	CTGGCCCGAAGAAC
EqIL10	ATGCCCCAGGCTGAGAAC	CGGAGGGTCTTCAGCTTTTCC	CCAGACATCAAGGAGCACG
EqIL1RA	AGTTGCTGGATACTTGCAAGAATCA	GAGTCCCAGGAATAGAGCATCAG	CATCTATCTTCTCTTGTAATTTA
EqINFγ	AGCAGCACCAGCAAGCT	TTTGCGCTGGACCTTCAGA	ATTGAGATTCCGGTAAATG
EqTLR2	Ec03818334_s1		
EqTLR4	Ec03468994_m1		

4.4 Results

Of 15 mares screened for susceptibility, 7 were identified as susceptible and used for this study. One mare was euthanized for non-related reasons during the study. One mare developed a bacterial infection that required treatment, causing a one cycle break between treatment cycles. None of the mares showed adverse reactions to the treatments.

Expression of IL1β was lower after treatment with dexamethasone ($P < 0.001$) and MCWE ($P = 0.04$) when compared to control (Figure 4.1). There tended to be an overall effect of treatment on IFNγ mRNA expression, but this reduction was not significant ($P = 0.079$) (Figure 4.2). No differences were detected in the mRNA expression of toll-like receptor (TLR)2, TLR4, IL1RA, IL6, and IL10 with either treatment (Fig. 4.3-4.7).

Figures 4.1-4.7: Endometrial mRNA expression of inflammatory genes in susceptible mares 6 hours after breeding with dexamethasone or MCWE. Treatments are compared to untreated susceptible mares 6 hours after breeding. Differing letters represent a significant difference in mRNA expression in treatment versus controls ($P < 0.05$).

Figure 4.1

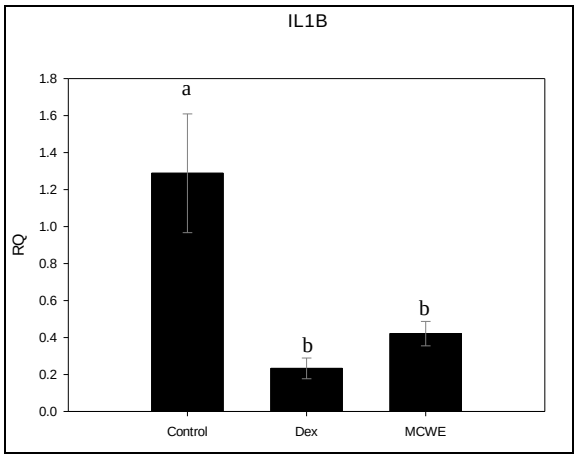


Figure 4.2

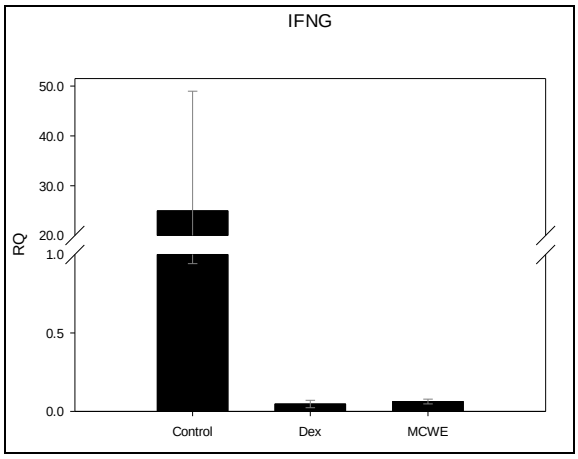


Figure 4.3

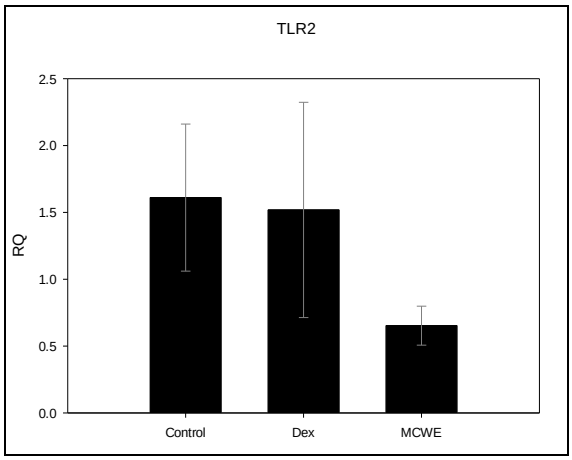


Figure 4.4

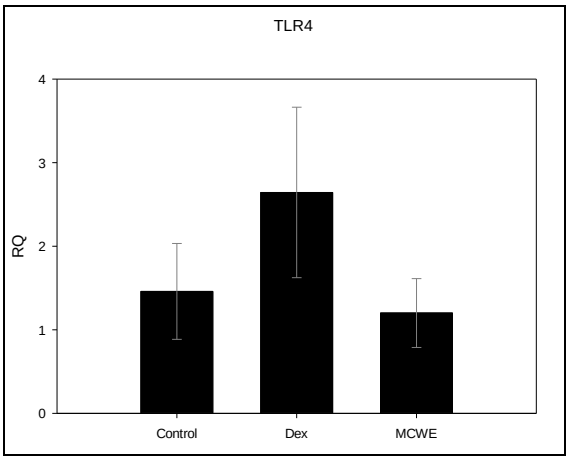


Figure 4.5

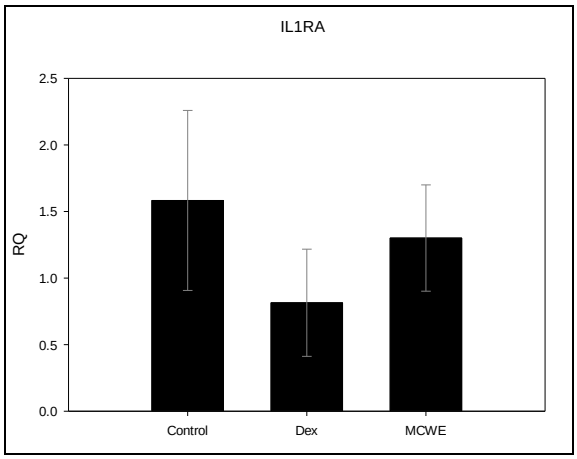


Figure 4.6

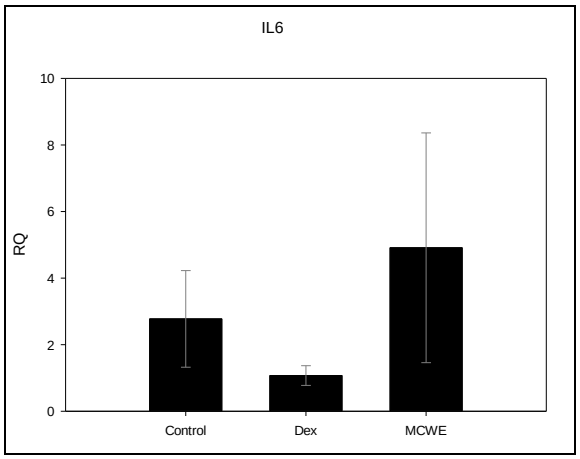
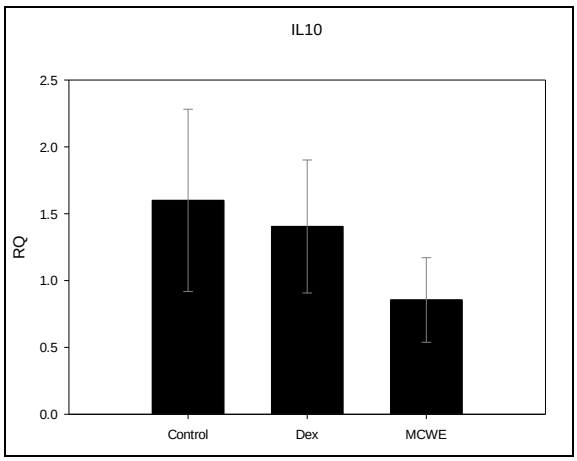


Figure 4.7



4.5 Discussion

The inflammatory response is a complex process involving many signaling cascades leading to the resolution of inflammation, and cytokines have a significant role in the recognition of pathogens and recruitment of inflammatory cells (Kuby 1992, Parham 2005). Cytokine signaling and the innate immune response during inflammation are well documented in other species and organ systems, however there are fewer studies focusing on these inflammatory pathways in the mare reproductive tract. The innate immune response is non-specific to antigen; however, specificities do exist for recognizing similar pathogen associated molecular patterns (PAMPs) (Aderem & Ulevitch 2000). Gram negative bacteria are recognized by TLR4, and the response to gram positive bacteria and mycobacterium has been reported to be initiated by TLR2 (Takeuchi *et al.* 1999, Aderem & Ulevitch 2000, Kannaki *et al.* 2011). Once the PAMPs are recognized, IL1 and IFNγ transcription are initiated, and their subsequent release leads to downstream release of inflammatory signals such as other inflammatory cytokines, leading to the activation and recruitment of inflammatory cells (such as PMNs) to the inflamed area. The results from this study did not detect an effect of MCWE on TLR2, which may suggest that the MCWE did not migrate to the endometrium, or that an upregulation of TLR2 due to MCWE occurred at a different time point than the 6 hour time point investigated. To the author's knowledge, it is unknown whether spermatozoa trigger activation of TLR2 and TLR4.

Four data points were removed as outliers for this experiment, and all four points came from the same mare. When looking at the IFNγ mRNA expression, the RQ value of the control sample from that mare was much higher than the other samples in that group,

leading to a greater SEM for the control samples. However, the data point was within the range of inclusion as defined by the study, and therefore was included in the data for IFNγ. When including the data point, the data were not normally distributed (even after data transformations) and were analyzed non-parametrically, to yield a P value of 0.079 for the effect of treatment. If the data point were excluded from the data (due to the fact that the mare was the sole outlier in the study), the data would be normally distributed, and dexamethasone would decrease mRNA expression (P = 0.02).

Immune stimulators (such as MCWE) act to prime the immune system. One type of MCWE, Bacillus Calmette-Guérin (BCG) derived from *Mycobacterium bovis* is used as a treatment for bladder cancer (Bohle 2000). BCG induced production of pro-inflammatory cytokines IL1, IL2, and TNF in the urine of patients treated for bladder cancer, with a peak of production 4-8 hours after treatment. By 24 hours, cytokine production returned to baseline levels (Bohle *et al.* 1990). The level of IL1β mRNA in the present study was decreased, not elevated, after treatment with MCWE. It is probable that levels returned to baseline 24 hours after treatment with MCWE, then continued to drop. Uterine samples were collected 6 hours after breeding, which was actually 30 hours after treatment with MCWE. Additionally, IL1β may have been decreased due to the MCWE priming the local immune response, thereby altering the cytokine response after the breeding challenge. Finally, a decrease in IL1β could lead to a decreased inflammatory response and a normal resolution of inflammation.

To mediate the inflammatory response, modulating cytokines are released, such as IL10 and IL1RA (Dinarello 1991, Duell *et al.* 2012). Corticosteroids act to supress the response of pro-inflammatory cytokines, chemokines, and other proteins associated with

inflammation such as iNOS, COX2, and adhesion molecules. In addition, corticosteroids upregulate transcription of immunomodulating cytokines such as IL10 and IL1RA (Barnes 1998). Although no effect of dexamethasone was observed on the inflammatory modulating cytokines in this study, IL1β mRNA expression was decreased after administration of dexamethasone. This is in agreement with another study that found that dexamethasone decreased IL1β mRNA expression in susceptible mares with experimentally induced bacterial endometritis (Christoffersen *et al*. 2012).

The suppression of IL1β after both treatments resembles the inflammatory modulating patterns observed in resistant mares after breeding. A previous study found that susceptible mares have a decreased production of the inflammatory modulating cytokines IL10, IL1RA, and IL6 when compared to resistant mares 6 hours after breeding (Woodward *et al.* 2011a). In this study, treatment had no effect on the immune modulating cytokines 6 hours after breeding, but did have a suppressive effect on pro-inflammatory cytokine response, possibly through a different pathway than via activating the immunomodulating cytokines. Interestingly, when investigating mRNA expression of these cytokines 3 hours after bacterial-induced endometritis in susceptible mares, expression of IL6 and IL10 was higher after treatment with dexamethasone when compared to untreated controls (Christoffersen *et al.* 2012). It is possible that these cytokines would have been differentially expressed in this study 3 hours after breeding, which may have led to the decrease in IL1β observed 6 hours after breeding.

Under the conditions of this study, an effect of treatment for many of the cytokines was not detected; however, the statistical power was low. An increased number of animals would improve the experimental power, reducing the chance of a Type 1

statistical error and yielding more insight into the effects of treatments in this group of mares. While the addition of several more mares in each group would have increased statistical power, it was feasibly not possible to do for this study.

4.6 Conclusion

In conclusion, the inflammatory cascades of inflammatory signals are complex and intertwined, with many points for regulation throughout. Treatment with immune modulation therapy has proven effective clinically in other studies; yet a complete understanding of the mechanisms behind these treatments remains a question. The results from this study show that these treatments may act to change the inflammatory cascade early on in the inflammatory process, especially with the IL1β pathways. Continued investigation into the mechanisms of these treatments will yield valuable information so that treatments can be improved in time, dosage, and frequency.

4.7 Acknowledgments

This work was supported by funds from the Gluck Fellowship in the Department of Veterinary Science, the Janet H. Koller Endowment for Equine Research, and Bioniche Life Sciences Inc. The authors thank Mr. Lynn Ennis, Mr. Kevin Gallagher, and the University of Kentucky's Maine Chance Farm for the management and care of the horses, the University of Kentucky Veterinary Diagnostic Laboratory for the use of their equipment and sample processing.

Chapter 5

An investigation of uterine nitric oxide production in mares susceptible and resistant to persistent breeding induced endometritis, and the effects of immunomodulation.

Woodward, E.M.[1], Christoffersen, M.[2], Campos, J.[1], Horohov, D.W.[1], Scoggin, K.E.[1], Squires, E.[1], Troedsson, M.H.T[1].

[1]The Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, 40546-0099, USA; [2]Department of Large Animal Sciences, Veterinary Reproduction and Obstetrics, University of Copenhagen, Dyrlaegevej 68, DK-1870 Frederiksberg C, Denmark

Will be submitted to Reproduction of Domestic Animals

5.1 Summary

Transient uterine inflammation after breeding is a physiological process necessary for clearance of bacteria and excess spermatozoa. In a subpopulation of mares, the inflammation fails to resolve in a timely fashion, resulting in a uterine environment that is detrimental to the conceptus. Nitric oxide (NO) is a potent smooth muscle relaxer involved in the inflammatory process, and has been implicated in reproductive pathologies in several species. The first objective of this study was to evaluate the presence of intrauterine NO and endometrial iNOS in mares susceptible or resistant to persistent breeding induced endometritis (PBIE) during the first 24 hours after breeding. Twelve mares were classified as susceptible (n = 6) or resistant (n = 6) to PBIE based on their clinical response to insemination with dead spermatozoa (intrauterine fluid

accumulation, uterine culture and cytology). All mares were inseminated over 5 estrous cycles and uterine secretions and endometrial biopsies were collected at one time point per cycle in randomized order before (0), and 2, 6, 12, and 24 hours after insemination. Uterine secretions were analyzed for nitric oxide (NO) production, and the presence of iNOS in the endometrial tissue was assessed using immunohistochemistry (IHC) and qPCR. Comparisons were made at each time point within treatments and between groups. Data were analyzed using two-way repeated measures ANOVA and significance was set at $P < 0.05$. A second experiment evaluated the effect of treatment with dexamethasone or mycobacterial cell wall extract (MCWE) on intrauterine NO production and endometrial iNOS mRNA expression. Six susceptible mares were inseminated over 3 estrous cycles with (1) killed spermatozoa without treatment (control), (2) killed spermatozoa with 50 mg dexamethasone iv at the time of breeding, or (3) MCWE iv 24 hours prior to insemination with killed spermatozoa. Six resistant mares were bred with killed spermatozoa as a control. Six hours after breeding, uterine biopsies and secretions were collected and processed as for Experiment 1. In Experiment 1, resistant mares had an increase of iNOS mRNA expression at 2 hours post breeding when compared to baseline ($P = 0.045$), 12 hours ($P = 0.014$), and 24 hours ($P = 0.001$). Susceptible mares had higher mRNA expression at 2 hours when compared to 6 hours ($P = 0.046$). No differences were observed in mRNA or protein expression of iNOS between resistant and susceptible mares at any time point. Resistant mares had a relatively steady amount of total intrauterine NO over the course of 24 hours, while susceptible mares had an increase over time, with a significantly higher increase in total NO than resistant mares at 6 ($P = 0.04$) and 12 hours ($P = 0.032$). In Experiment 2, no differences were observed for iNOS

mRNA expression after treatments. Susceptible mares had an increased production of intrauterine NO when compared to resistant mares (P = 0.008), and intrauterine NO was decreased after treatment with MCWE (P = 0.047). In conclusion, intrauterine NO is increased in mares susceptible to PBIE, and treatment with the immune modulator MCWE reduces total intrauterine NO production.

5.2 Introduction

A local inflammatory response is a physiological event after breeding, which aids to clear excess semen, bacteria, and debris from the reproductive tract (Troedsson 2006). In reproductively sound mares, the inflammation subsides within 48 hours, however a subset of mares fails to clear the inflammation. It has been suggested that PBIE is caused in part by impaired myometrial activity, resulting in delayed uterine clearance.

Efficient uterine expulsion of fluid and debris involves contractions of the myometrium (Hirsbrunner *et al.* 2006, Hirsbrunner *et al.* 2010). After bacterial challenge, both susceptible and resistant mares had an increase in myoelectrical activity. However, the increase in the susceptible mares was delayed by 2 hours, and while susceptible mares were able to reach the same level of activity as the resistant mares, they had a sharp decline in activity 6 hours post challenge and dropping below baseline levels 12 hours post challenge, indicating myometrial relaxation (Troedsson *et al.* 1993b). Failure of uterine clearance was further investigated in 1994 using scintigraphy when researchers observed that susceptible mares retained more radiocolloid 2 hours after insemination than resistant mares (LeBlanc *et al.* 1994). It was concluded that myometrial contractility

defects may be a major culprit in the pathogenesis of PBIE, and researchers began to investigate factors influencing the functionality of the myometrium.

Nitric oxide (NO) is a potent cell signaling molecule and known smooth muscle relaxer occurring in many body systems in both physiological and pathological states (Hou *et al.* 1999, Tripathi 2007, Angulo *et al.* 2010, Eleftheriadis *et al.* 2010, Gupta *et al.* 2010, Zanetti *et al.* 2010). During inflammation, inflammatory signals such as interleukin (IL)1 and interferon gamma (IFNγ) trigger a cascade of events that lead to the initiation of inducible nitric oxide synthase (iNOS), which produces large amounts of NO (Rosselli *et al.* 1998, Saxena *et al.* 2000, Tripathi 2007, Li *et al.* 2010). The increased NO aids in the removal of pathogens, but can also be cytotoxic (Macmicking *et al.* 1997, Tripathi 2007). Another effect of NO is smooth muscle relaxation. Myometrial tissue *in vitro* was unable to respond to electrical stimulus in the presence of NO (Liu *et al.* 1997). An *in vivo* study found that mares susceptible to PBIE have increased iNOS activity and NO production when compared to resistant mares 13 hours after insemination (Alghamdi *et al.* 2005b). The authors suggested that contractility deficiencies in susceptible mares (Troedsson *et al.* 1993b) may be associated with increased NO production (Alghamdi *et al.* 2005b).

The first objective of this study was to evaluate NO production and iNOS expression at several time points within the first 24 hours after challenge with killed spermatozoa. The hypotheses were that (1) intrauterine NO production and iNOS expression change in mares at varying time points within 24 hours after insemination, and (2) that mares susceptible to PBIE have different levels of intrauterine NO and endometrial iNOS than mares resistant to PBIE within the first 24 hours of breeding. In

addition, the second objective was to determine the effects of immune modulators on intrauterine NO production and iNOS mRNA expression *in vivo*.

5.3 Materials and Methods

5.3.1 Animals

Mares of mixed breeds, age, and breeding history were kept on pasture supplemented with grain and hay, and were provided with water and salt *ad-libitum*. Semen was collected from 3 stallions with similar housing conditions to that of the mares. The experiment was approved by the University of Kentucky's IACUC (protocol numbers 2009-0455 and 2009-0602).

5.3.2 Preparation of sperm for insemination

Freeze-killed spermatozoa were prepared by removing the seminal plasma from ejaculates using centrifugation, resuspending the spermatozoa pellet in milk based extender (EquiPro®, Minitube, Verona, WI) in aliquots of 1×10^9 spermatozoa in 30 mL of extender, followed by at least 2 freeze (−20°C)/thaw (room temperature) cycles of the aliquots. This method has previously shown to be an effective method to kill spermatozoa (unpublished data).

5.3.3 General experimental procedure for the selection of mares

Mares were examined for susceptibility to PBIE based on endometrial histology and the results of a spermatozoa challenge. Endometrial biopsies (approximately 300 mg of tissue) were obtained during diestrus using an alligator jaw biopsy instrument, fixed in

10% formalin, sectioned at 5 μm and stained with hematoxylin and eosin. Each biopsy was examined for periglandular fibrosis, inflammatory cells, glandular distribution and lymphatic lacunae, then graded according to Kenney and Doig (Kenney & Doig 1986). Mares with scores of I or IIA were considered potentially resistant to PBIE, while those with scores of IIB or III were potentially susceptible to PBIE (Troedsson *et al.* 1993a).

After selection as potentially resistant or susceptible to PBIE, the mares' reproductive tracts were observed regularly for signs of estrus and pending ovulation using transrectal ultrasonography. Estrus was defined by the presence of uterine edema, a relaxed cervix, and at least one follicle 35 mm or larger in diameter in the absence of a *corpus luteum*. Once estrus was detected, mares were evaluated for the presence of intrauterine inflammatory cells using a cytobrush (Minitube) or low volume lavage to obtain samples, and for intrauterine bacteria using a double-guarded swab (Minitube) to obtain samples, and then were inseminated with freeze-killed spermatozoa (prepared as above). For the cytological evaluations, inflammation was defined as more than 2 neutrophils per 5 fields at ×400 magnification. Swab samples were incubated at 37°C for 24 hours and evaluated for growth. Mares were challenged with an intrauterine inoculation of 1×10^9 freeze-killed stallion spermatozoa (prepared as above). Only mares with negative cytology and no bacterial growth prior to insemination were considered for the experiment.

Potentially susceptible mares were evaluated 96 hours after insemination and were confirmed as a susceptible mare if they had 1) positive cytology, and 2) uterine fluid retention. Potentially resistant mares were assessed 48 hours after breeding, and those with 1) negative cytology and culture in addition to 2) no uterine fluid retention were

confirmed as resistant mares (Troedsson & Liu 1991, Alghamdi *et al.* 2005b). All mares not meeting the criteria for classification as susceptible or resistant were excluded from the experiments.

5.3.4 Experiment 1

Six resistant (aged 5-15 years) and 6 susceptible (aged 10-27 years) were used for this experiment. Over 5 subsequent cycles, estrus was induced through administration of prostaglandin, (Lutalyse®, 7.5mg, Pfizer, New York, NY). Once estrus was detected, mares were evaluated for cytological and bacterial findings, and then inseminated with 1×10^9 dead spermatozoa (as described above). Only estrous cycles with negative cytology and culture were used for the experiment; if mares were positive for cytology or culture at the time of insemination, they were treated and insemination attempted again during the subsequent cycle. Samples were collected after insemination at one time point per cycle in randomized order. Time points were: 0 (before insemination), 2, 6, 12, and 24 hours after insemination.

5.3.5 Experiment 2

Six susceptible mares (15-24 years) and 6 resistant mares (3-16 years) were identified and used for the experiment. Over 3 subsequent cycles, estrus was induced in the susceptible mares through administration of prostaglandin, (Lutalyse®, 7.5mg, Pfizer). Once estrus was detected with an ovarian follicle of 35 mm in size or larger, mares were administered one of the following treatments: 1) killed spermatozoa in extender (prepared as described above) 2) 50 mg dexamethasone iv and insemination

with killed spermatozoa (prepared as described above) and 3) 1.5 mg MCWE (Settle™, Bioniche, Bogart, GA) iv when a follicle ≥ 30 mm and uterine edema was present, and insemination with killed spermatozoa (prepared as described above) 24 hours after treatment. Uterine tissue and secretions were collected 6 hours after treatments. Treatment schedules for the susceptible mares were randomized, and only estrous cycles with negative cytology and culture were used for the experiment. For the resistant mares, once estrus was detected with a follicle 35 mm or larger, mares evaluated for cytological and bacterial findings, and then inseminated with 1×10^9 dead spermatozoa (as described above). Uterine tissue and secretions were collected 6 hours after insemination. As with the susceptible mares, only estrous cycles with negative cytology and culture were used for the experiment; if mares were positive for cytology or culture at the time of insemination, they were treated and insemination was attempted again during the following cycle.

5.3.6 Sample collections for Experiments 1 and 2: uterine secretions

To collect uterine secretions, a sterile tampon was inserted manually into the uterus as previously described (Alghamdi *et al.* 2005b). Briefly, a sterile cotton tampon was manually inserted into the uterus with a gloved hand, and then the uterus was massaged via rectal palpation intermittently for 5 minutes. Using a sterile speculum, the tampon was removed into the speculum and then out of the mare to avoid contamination with vaginal fluids. To collect remaining secretions, the uterus was lavaged with 250 mL of Lactated Ringer's solution after tampon removal. The tampons and uterine lavage samples were centrifuged at 1000 × g for 10 minutes, and the supernatants were stored at -20°C until further processing.

5.3.7 Sample collections for Experiments 1 and 2: endometrial tissue

Two uterine biopsies were collected; one was fixed in 10% formalin for immunohistochemistry (Experiment 1 only), and the other was either flash frozen in liquid nitrogen and stored at −80°C until further processing, or stored in RNAlater® (Applied Biosystems, Carlsbad, CA) overnight at 4°C, then moved to −20°C for storage until further processing (Experiments 1 and 2).

5.3.8 Determination of intrauterine nitric oxide

To determine the amount of intrauterine nitric oxide, total uterine fluid (V_1) was calculated using the equation $C_1V_1 = C_2V_2$. Protein concentrations were measured in the uterine secretions and lavage samples with a BCA Protein Assay® kit (Thermo Scientific, Kalamazoo, MI) in the uterine secretions collected with the tampon (C_1) and the uterine lavage samples (C_2). Total uterine fluid (V_1) was determined with the protein concentrations from the uterine samples, and V_2 was calculated after taking into consideration the addition of the 250 mL of Lactated Ringers into the uterus ($250 + V_1$). Once initial intrauterine fluid volume was determined, a commercially available NO assay (Bioxtech® Nitric Oxide Assay, OxisResearch™, Portland, OR) was used to determine the total amount of NO in the uterine secretions from the tampons. The kit works by measuring the stable metabolites nitrate and nitrite (which are produced as NO is degraded). Nitrate was converted to nitrite by nitrate reductase, and total nitrite was measured and compared to known concentrations on a standard curve. Samples were run

in duplicate in 96 well plates, and using a spectrophotometer (Benchmark Plus Microplate Reader™, Bio-Rad, Hercules, CA) were read at an absorbency of 540 nm. NO concentrations were calculated against a standard curve, and then multiplied by the total initial volume of uterine secretions (V_1) to determine total intrauterine amount of NO. Percentage change in NO from 0 hours was compared at each time point with pairwise comparisons with SigmaStat© (Systat Software, Inc.). Outliers were defined as ± 2 SD from the mean and removed (2 of 50 data points, 4%) from statistical analyses.

5.3.9 Immunohistochemistry

Tissue from Experiment 1 were fixed in formalin, embedded in paraffin, and were then used for immunohistochemistry. Slides were sectioned at 5 uM and stained with an anti-human monoclonal antibody for iNOS raised in mouse (1:25 dilution, R and D Systems, Minneapolis, MN). Slides were processed with the Leica BOND-MAX™ system (Leica Microsystems, Buffalo Grove, IL). Negative controls were prepared in the absence of primary antibody, and positive controls were prepared using mouse recombinant iNOS (Western Blot) and lung, liver, and small intestinal tissue from a sick foal infected with *Lawsonia intracellularis*. Slides were observed at ×400 magnification and 3 representative fields from each slide were used for staining analysis. Images were analyzed using ImmunoRatio© (Jorma Isola and Vilppu Tuominen, Institute of Biomedical Technology, University of Tampere, Finland) a software program that calculates the percentage of diaminobenzidine (DAB) stained cells from total cells (Tuominen *et al.* 2010). Each field was analyzed with the program and the percentage staining of DAB was averaged from the 3 slides from each sample. Data were analyzed

using SigmaStat© (Systat Software, Inc.) with two-way repeated measures ANOVA tests. Data were log10 transformed for normal distribution, and outliers were defined as ± 2 SD from the mean and were removed from statistical analyses (4 of 58 data points; 6.8%). Missing data points were automatically calculated by the software using a general linear model **(**Sigma Stat User Guide, p. 379). Comparisons were made between groups at each time point, and between time points within groups. Significance was set to $P < 0.05$.

5.3.10 qPCR analysis

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated with a commercially available kit (DNA-free™, Applied Biosystems, Carlsbad, CA), then analyzed for quality and quantity using a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA). 1.5 µg of RNA in 41.5 µL ddH_2O was reverse transcribed in a reaction using AMV Reverse Transcriptase (0.5 µL;10 U/µL), 5× RT Buffer (16 µL), RNAsin® (1 µL; 40 U/µL), MgCl (16 µL; 25 mM), dNTP (4 µL;10 mM), and Oligo(dT) Primer (1 µL; 500 µg/mL) (all reagents from Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 minutes, then 95°C for 5 minutes. cDNA was diluted 1:1 with ddH_2O, and qPCR for each sample was performed using 4.5 µL of cDNA, 5 µL of Sensimix™ II (Bioline, Tauton, MA, USA), and 0.5 µL of a custom dual hydrolysis primer/probe set for iNOS (Applied Biosystems). Reactions were performed in duplicate, and using the 7900HT Fast Real-Time PCR System (Applied Biosystems), were incubated at 95°C for 10 minutes, followed by 45

cycles of 95°C for 15 seconds and 60°C for 60 seconds. PCR efficiencies were calculated using LinRegPCR (version 7.0). β-actin was used as the reference gene, and results are expressed as mean relative quantification values (RQ) which were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), with the calibrator as the mean cycle threshold (ΔC_T) value of the 0 hour collections from the resistant mares for Experiment 1. For the comparison between resistant and untreated susceptible mares in Experiment 2, the calibrator was the mean cycle threshold (ΔC_T) value of the resistant mares 6 hours post breeding. Finally, for comparisons between treatments in Experiment 2, the calibrator was the mean cycle threshold (ΔC_T) value of the untreated susceptible mares 6 hours post breeding. Data in Experiment 1 were analyzed using SigmaStat© with two-way repeated measures ANOVA tests. Data were square root transformed for normal distribution, and outliers were defined as ± 2 SD from the mean and were removed from statistical analyses (1 of 58 data points removed; 1.7%). Missing data points were automatically calculated by the software using a general linear model **(**Sigma Stat User Guide, p. 379). Comparisons were made between groups at each time point, and between time points within groups. Significance was set to $P < 0.05$. In Experiment 2, data were log10 transformed for normal distribution, and outliers were defined as ± 2 SD from the mean and were removed (1 of 58 data points for the total intrauterine NO production, and no outliers for the iNOS mRNA expression). Data were analyzed using SigmaStat©, and comparisons were made between treatments for the susceptible mares using a one way ANOVA, and between the control samples for resistant and susceptible mares using pairwise comparisons. Significance was set to $P < 0.05$.

5.4 Results

5.4.1 Experiment 1

Resistant mares had an increase of iNOS mRNA expression 2 hours post breeding when compared to baseline (P = 0.045), 12 (P = 0.014), and 24 hours (P = 0.001) (Figure 5.1). In addition, expression of iNOS mRNA was higher at 6 hours when compared to 24 hours (P = 0.005) in resistant mares. Susceptible mares had significantly higher mRNA expression 2 hours after breeding when compared to 6 hours (P = 0.046) (Figure 5.1). No differences were observed in mRNA expression of iNOS between resistant and susceptible mares at any time point (Figure 5.1). No differences were observed for time point or group for iNOS protein expression (Figure 5.2). Resistant mares had a relatively steady amount of total intrauterine NO over the course of 24 hours. However, susceptible mares had an increase over time, with a significantly higher increase in total NO than resistant mares at 6 (P = 0.04) and 12 hours (P = 0.032) (Figure 5.3).

5.4.2 Experiment 2

No differences were observed in mRNA expression of iNOS between groups or treatments (Figure 5.4). Total intrauterine NO was greater in untreated susceptible mares when compared to resistant mares (P = 0.008) (Figure 5.5). In the susceptible mares, dexamethasone had no effect on intrauterine NO, although total NO was decreased in the uterus when treated with MCWE (P = 0.047) (Figure 5.6).

Figure 5.1: iNOS mRNA expression in resistant and susceptible mares after breeding. RQ values describe fold changes in each mare compared to the ΔC_T value of resistant mares at 0 hours. Data presented in graphs are the raw RQ values, although data was square root transformed for statistical analysis. Error bars report standard error of the mean. Differing letters over the bars within each group represent significant differences (capital letters for susceptible; lowercase for resistant). Significance set to $P < 0.05$.

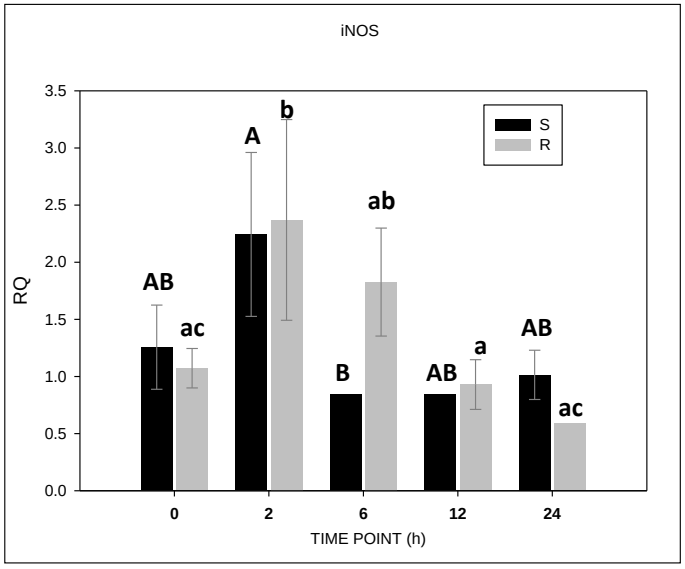


Figure 5.2: Percentage of positively stained endometrial cells for iNOS protein in resistant and susceptible mares at different time points after breeding. Significance set to $P < 0.05$.

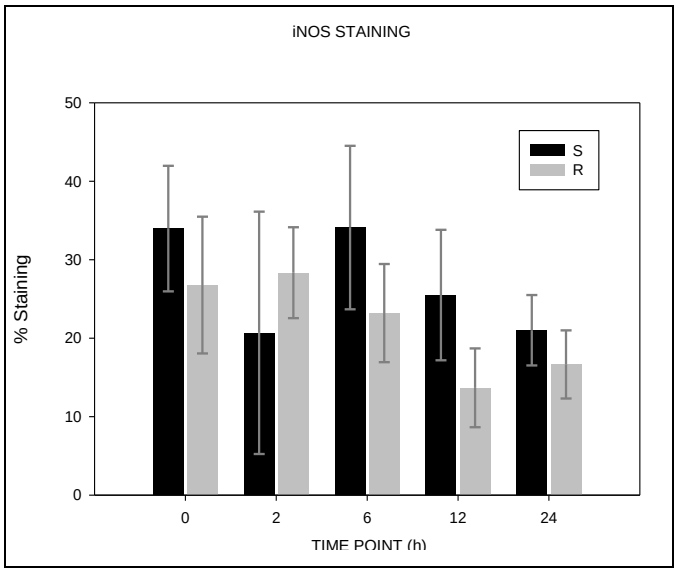


Figure 5.3: Percentage change in intrauterine NO production over time in resistant and susceptible mares after breeding. An asterisk (*) denotes significance ($P < 0.05$)

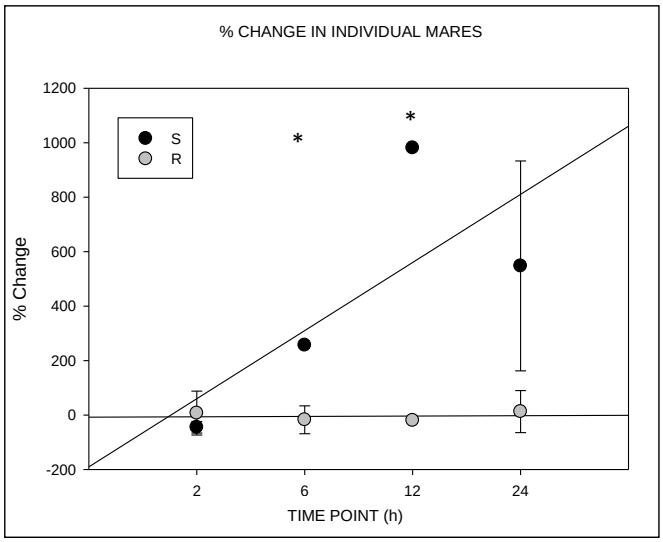


Figure 5.4: The effect of immunomodulators on iNOS mRNA expression in susceptible mares 6 hours post breeding. RQ values describe fold changes in each mare compared to the ΔC_T value of control breedings in the mares. Error bars report standard error of the mean. Significance set to $P < 0.05$.

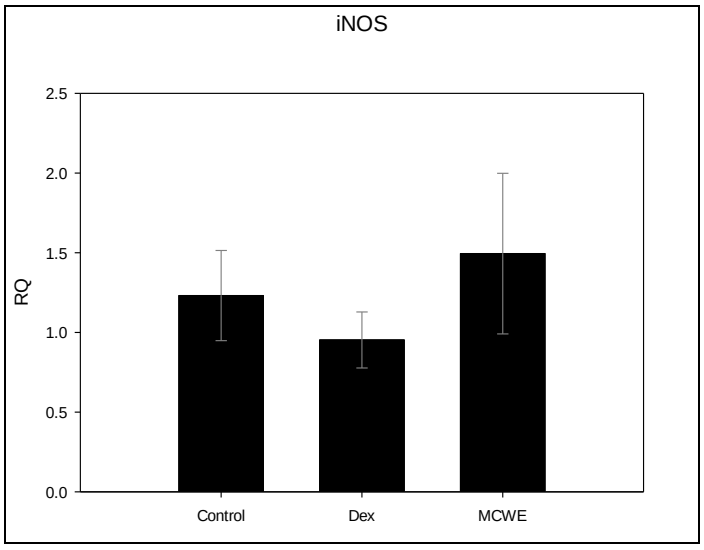


Figure 5.5: Total intrauterine NO in untreated susceptible and resistant mares 6 hours after breeding. Error bars report standard error of the mean. Differing letters represent significant differences. Significance set to $P < 0.05$.

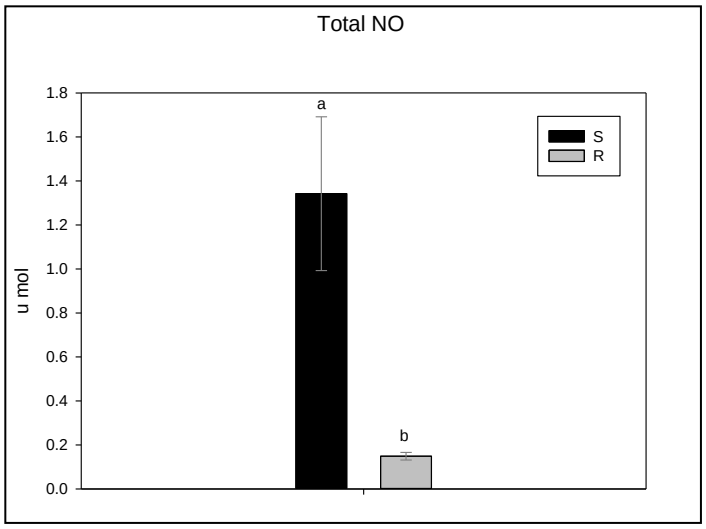
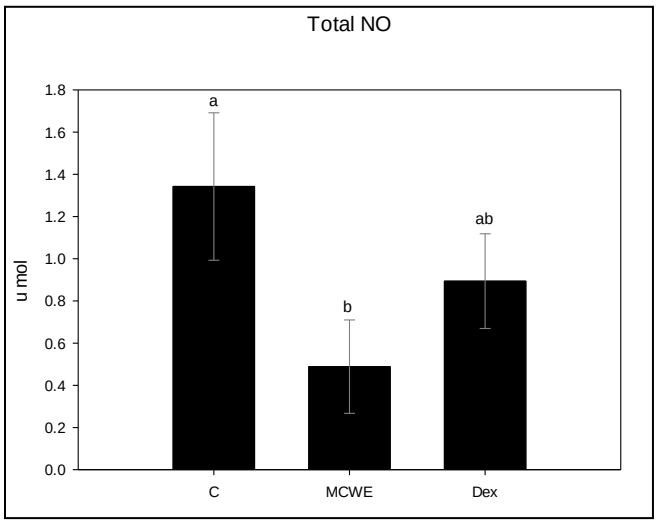


Figure 5.6: The effect of immunomodulators on total intrauterine NO in susceptible mares 6 hours post breeding. Error bars report standard error of the mean. Differing letters represent significant differences. Significance set to $P < 0.05$.



5.5 Discussion

This study investigated the role of NO in PBIE at the transcriptional, translational, and functional levels. The pattern of iNOS mRNA expression observed in Experiment 1 is similar to those observed in a previous study investigating inflammatory cytokine mRNA expression, where both pro- and anti-inflammatory cytokines peaked in expression 2 to 6 hours post challenge (Woodward *et al.* 2011a). The study observed that at 6 hours post breeding, resistant mares had an increase in the inflammatory modulating cytokines, but not the pro-inflammatory cytokines when compared to the susceptible mares. It was concluded that susceptible mares had a deficiency in the immune modulatory response. Under the conditions of this study, no differences were detected in iNOS mRNA or protein expression between susceptible and resistant mares at any time point (in contrast to previously described findings 13 hours after challenge) (Alghamdi *et*

al. 2005b). It is unclear as to why the two studies yielded different results for IHC and qPCR, however, it should be noted that the earlier study utilized a standard PCR technique while the current study used qPCR. Additionally, for the IHC analysis, different antibodies were used for the two studies, although both were validated to detect iNOS with a Western Blot.

Total intrauterine NO increased over time in the susceptible mares, while in the resistant mares, levels did not vary significantly in Experiment 1. The results from Experiment 2 were similar in that susceptible mares had an increased intrauterine NO production compared to resistant mares. These results support those found by previous studies that observed an increased intrauterine NO production in susceptible mares 13 hours after breeding (Alghamdi *et al.* 2005b). From the conclusions made in these studies, it is evident that the presence of intrauterine NO differs between resistant and susceptible mares after breeding, however it remains a question as to if NO is a cause or effect of PBIE and myometrial dysfunction.

There are a variety of approaches for the treatment of PBIE, with variations in dose, frequency, duration, and administration route (Combs *et al.* 1996, Watson 2000, Bliss & Campbell 2002, Hurtgen 2006, Liu & Troedsson 2008, LeBlanc 2010). Pregnancy rates were improved in mares treated with 5 doses of predinisolone acetate in mares with a history of fluid retention after breeding (Dell' Aqua *et al.* 2006). Other researchers observed a reduction in clinical signs of PBIE in susceptible mares treated with dexamethasone (Bucca *et al.* 2008). Dexamethasone acts to reduce IL1 activity, which (in addition to other pro-inflammatory cytokines) is an inducer of iNOS (Persichini *et al.* 2006). While IL1β mRNA expression was shown to be decreased 6 hours after

breeding in susceptible mares treated with dexamethasone in the experiment from Chapter 4, the results from Experiment 2 did not show an effect of dexamethasone on iNOS or NO production. These findings suggest that NO production is affected by dexamethasone at another time point, or that NO production in PBIE may be regulated by mechanisms other than IL1 pathway.

Mycobacterium cell wall extract is an immune stimulator used to treat cancer (Filion & Phillips 2001, Yuksel *et al.* 2011), respiratory disease, and as a vaccine adjuvant (Berinstein *et al.* 1991). In the mare, MCWE decreased the incidence of endometritis induced with a bacterial challenge (Rogan *et al.* 2007). In addition, MCWE was shown to decrease levels of pro-inflammatory cytokines in susceptible mares towards that of resistant mares 24 hours after breeding (Fumuso *et al.* 2003b, Fumuso *et al.* 2007). In this study, treatment with MCWE reduced total NO production when compared to non-treated controls. MCWE acts to prime the immune response, and it is possible that inflammatory signals and cells were initiated earlier with treatment versus than without treatment. Therefore, after treatment, the uterus was better prepared to respond to inflammation, leading to a more controlled response to spermatozoa, and less NO production.

The biological lifetime of NO is affected by cell uptake. NO freely diffuses through cell membranes, and the closer NO is to cells, the faster it is removed from solution. If susceptible mares have an increase in intrauterine fluid as a result of inflammation, then the increased volume in susceptible mares permits for a longer biological life in the uterus, as NO needs to travel further to reach the cells (Thomas *et al.* 2001). It is unclear as to whether or not NO is a major cause or an effect of persistent

endometritis. It is well documented that NO is an inflammatory byproduct, however, as a smooth muscle relaxant, it is possible that NO buildup could cause myometrial relaxation, leading to decreased clearance through the lymphatic ducts and cervix. This may lead to increased concentration and total NO, which may in turn lead to defective myometrial activity. In conclusion, the NO system plays a part in PIBE, although its role in the disease is still being understood.

5.6 Acknowledgements

This work was supported by funds from the Gluck Fellowship in the Department of Veterinary Science, the Janet H. Koller Endowment for Equine Research, and Bioniche Life Sciences Inc. The authors would like to thank Mr. Lynn Ennis, Mr. Kevin Gallagher and the University of Kentucky's Maine Chance Farm for the management and care of the horses, the University of Kentucky Veterinary Diagnostic Laboratory for the use of their equipment and sample processing, and Dr. Gabriel Davolli and Ms. Pauline Peugnet for their technical assistance.

CHAPTER 6

General Discussion

Persistent breeding-induced endometritis (PBIE) is a costly cause of infertility in mares, and identifying problem mares prior to the start of the breeding season is critical for efficiently producing a foal. Factors predisposing mares to persistent endometritis are: advanced age (Ricketts & Alonso 1991, Carnevale & Ginther 1992, Zent *et al.* 1998, Barbacini *et al.* 2003) multiparity, poor perineal conformation (Hemberg *et al.* 2005), and decreased endometrial quality (Troedsson *et al.* 1993a). The first objective of this project was to confirm correlations between susceptibility to PIBE with advanced age and endometrial biopsy score. The results from the experiments in Chapter 2 confirmed that mares with a poor endometrial biopsy quality or advanced age had a higher incidence of PBIE than younger mares, or mares with good biopsy scores. In addition, an attempt was made to classify mares as susceptible or resistant to PBIE, and to compare the status of the mares from one season to the next. Using strict criteria, mares were grouped as resistant or susceptible to PBIE, however many mares fell into an intermediate classification where they qualified as neither susceptible nor resistant. Furthermore, mares' classification changed from year to year, with most mares moving towards susceptibility with time.

These results suggest that a uterine biopsy score can be useful in predicting PBIE. The progression towards susceptibility appears to be gradual, since none of the mares changed all the way from resistant to susceptible or from susceptible to resistant, and rather, were classified as intermediate. Regardless, following a mare's uterine health can help to identify problem mares prior to the start of the breeding season.

Recently, the focus of persistent endometritis has shifted to inflammatory gene expression within the uterus. Previous studies have investigated inflammatory cytokine expression in the endometrium 24 hours after breeding (Fumuso *et al.* 2003b, Fumuso *et al.* 2006, Fumuso *et al.* 2007), however, studies investigating mechanical aspects of delayed uterine clearance (Troedsson & Liu 1991, LeBlanc *et al.* 1994) suggest that uterine function differs in susceptible and resistant mares earlier than that. The objective of the experiment in Chapter 3 was to describe endometrial inflammatory cytokine mRNA expression at selected time points within the first 24 hours after breeding. The results from the study suggest that 6 hours after breeding is a critical time to set up the mechanism for uterine response and clearance. When compared to resistant mares, susceptible mares seemed to have a defect in the inflammatory modulating portion of the immune response, while the pro-inflammatory cytokine mRNA expression was similar for resistant and susceptible mares.

There are several preventative and responsive treatments for PBIE. Immunomodulatory treatments are currently used in veterinary practice, but more studies focusing on immunomodulation for PBIE are needed. The objective of the experiment in Chapter 4 was to evaluate the impact of dexamethasone and mycobacterial cell wall extract (MCWE) on endometrial cytokine mRNA expression in susceptible mares 6 hours after breeding. Susceptible mares treated with dexamethasone and MCWE had a decrease in the pro-inflammatory cytokine interleukin (IL)1 mRNA expression 6 hours after breeding when compared to untreated controls. During the initiation of inflammation, IL1 is one of the primary pro-inflammatory cytokines involved with the response, and decreasing expression may help to reduce uterine inflammation. The results suggest that

susceptible mares have a decrease in the ability to mount an anti-inflammatory response (to keep pro-inflammatory signals in check). Therefore, it is possible immunomodulatory treatments can substitute for the lack in inflammation modulation in susceptible mares.

Nitric oxide (NO) is a potent cell-signaling molecule involved with inflammatory response. Previous work in horses and other species has demonstrated that mares susceptible to PBIE (Alghamdi *et al.* 2005b) and cows with post partum endometritis (Li *et al.* 2010) have an increased accumulation of intrauterine NO. In addition, studies investigating the effects of NO on myometrial relaxation have demonstrated that NO reduces myometrial contractility *in vitro* (Liu *et al.* 1997). The objective of the first experiment in Chapter 5 was to determine the intrauterine production of NO and the activity of iNOS at the transcriptional and translational levels in resistant and susceptible mares at different time points post breeding. Total intrauterine NO increased in susceptible mares over time, while resistant mares did not exhibit a marked change in intrauterine NO accumulation. This data supports the previous reports on NO in problem mares and cows, but further studies are needed to investigate if NO is a major contributor to the cause, or is solely an effect of PBIE. To investigate the impact of treatment on the role of intrauterine NO, a second experiment in Chapter 5 investigated the effects of treatment with dexamethasone and MCWE on intrauterine NO production and iNOS mRNA expression 6 hours after breeding. The study confirmed the findings from the first experiment in that susceptible mares had an increase in intrauterine NO production when compared to resistant mares. In addition, treatment with MCWE decreased total intrauterine NO production when compared to untreated control breedings in susceptible mares.

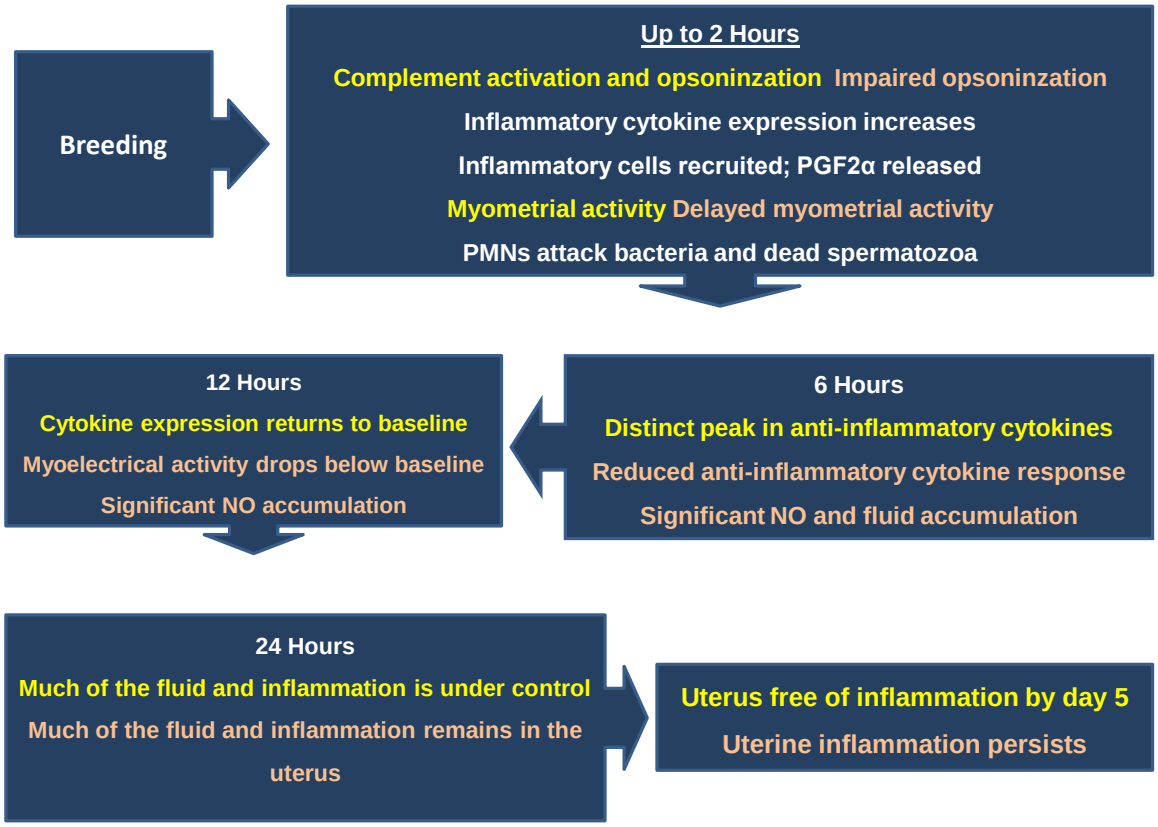
These experiments yield a better understanding of the progression of PBIE within the first hours after breeding, and how treatments with immunomodulation adjusts inflammatory parameters. Using the findings from these studies, an updated proposed mechanism of the inflammatory uterine response to breeding progression of the disease is as follows (Figure 6.1):

Shortly after breeding, spermatozoa and bacteria are opsonized, with a preferential opsonization towards dead and damaged spermatozoa (Alghamdi & Foster 2005a, Doty *et al.* 2011). The presence of spermatozoa and bacteria leads to cytokine release and subsequent recruitment of polymorphonuclear neutrophils (PMNs). As the PMNs migrate through the endometrium, prostaglandin (PGF)2α is released, causing uterine contractions. Another factor that may contribute to uterine contractions shortly after breeding is oxytocin release in response to breeding stimuli (Madill *et al.* 2000). Inflammatory cytokine release rises by 2 hours after breeding, continuing the signals for the influx of inflammatory cells. Uterine contractions continue to aid in the expulsions of excess semen and debris, and fluid is also removed via lymphatic drainage (LeBlanc *et al.* 1995). At around 6 hours after breeding, expression of inflammatory signals peaks. A strong anti-inflammatory response keeps the pro-inflammatory response in check. At this time, the majority of spermatozoa have made it to the oviduct (Brinsko *et al.* 1991). By 12 hours after breeding, inflammatory cytokine mRNA expression and uterine activity (Troedsson *et al.* 1993b) are decreasing towards baseline levels. By 24 hours, much of the inflammatory byproducts have been removed from the uterus, and the uterus is primed to be fully recovered and returned to baseline state before day 5, at which point

the conceptus arrives into the uterus (Oguri & Tsutsumi 1972, Betteridge *et al.* 1982, Troedsson *et al.* 1995b, Troedsson 1999).

In the susceptible mare, pathogens may not be opsonized as efficiently as the resistant mare (Watson *et al.* 1987, Troedsson *et al.* 1993c). As with the resistant mare, inflammatory cytokine response increases at 2 hours, but uterine activity has a delay in the onset of activity (Troedsson *et al.* 1993b, LeBlanc *et al.* 1994). Recruitment of PMNs triggers the release of PGF2α, but uterine contractions between circular and longitudinal myometrial layers may be less synchronized and therefore less effective in removing fluid (LeBlanc *et al.* 1994). Lymphatic draining is reduced due to uterine scarring and fibrosis. Six hours after breeding, inflammatory cytokine expression is at its peak, however, anti-inflammatory cytokine response is diminished (compared to the normal response), leaving the pro-inflammatory response unchecked. By 12 hours, the acute inflammatory cytokine responders have decreased, however, inflammation still remains. The buildup of the inflammatory byproduct NO contributes to the loss of myometrial activity below baseline levels (Troedsson *et al.* 1993b). Blood flow is reduced due to degenerative changes in the endometrium, so despite the increased amounts of NO, inflammation is not cleared as easily. The residual inflammation and continued migration of PMNs through the endometrium leads to additional degenerative changes, which further complicate the mares' ability to clear inflammation successfully. If left untreated, the susceptible mare may not have the ability to clear inflammation by day 5, and the conceptus will not survive in the inflamed uterine state.

Figure 6.1: Proposed sequences of events occurring after breeding in susceptible and resistant mare including inflammatory gene expression and NO accumulation. Yellow text refers to resistant mares, orange text to susceptible mares, and white text to both resistant and susceptible mares.



The proposed mechanism of action for the treatment with dexamethasone and MCWE are as follows:

Both dexamethasone and MCWE act as a substitute for the anti-inflammatory response lacking in susceptible mares 6 hours after breeding. As a result, the pro-inflammatory response is kept in check (especially the pro-inflammatory cytokine IL1β), allowing for the continued clearance of inflammation. Inflammation and NO buildup is reduced, allowing for the continued myometrial functionality and mechanical clearance of fluid. There is less residual inflammation, and therefore the progression of degenerative changes in the endometrium is slowed. The mare is able to clear the inflammation by day 5 and the chance of survival of the conceptus is increased.

Future directions of research into PBIE should focus on the pathogenesis of the disease. When investigating the pathogenesis of PBIE, a mass analysis of gene expression (such as a microarray or other sequencing technologies) in susceptible versus resistant mares would give further insight into processes occurring in the endometrium during inflammation. With answers to this question, further research could focus on areas of differentially expressed genes. Investigation of these genes on a translational level could reveal which genes are directly affecting inflammatory processes. After identifying differential gene expression and function, studies could be performed investigating gene regulation and regulatory factors. Additionally, investigating the effects of immunomodulatory treatments on these regulatory factors would increase the knowledge of the biological mechanism of action of these treatments.

In conclusion, the events occurring during the uterine inflammatory response to breeding are complex and intertwined. With all factors combined, susceptibility to PBIE

seems to be a function of age, endometrial quality, and breeding history. Although endometritis is not considered to be persistent until more than 48-72 hours after breeding, it appears that the inflammatory changes differentiating resistant and susceptible mares occurs within the first hours after breeding. Treatment with immune modulation therapy has proven effective clinically, and the findings from these studies help to explain the targeted inflammatory mechanisms of immunomodulation. Persistent endometritis remains a threat to equine fertility, and continued research will yield valuable information so that treatment strategies can be improved in time, dosage, and frequency.

REFERENCES

Aderem A & Ulevitch RJ 2000. Toll-like receptors in the induction of the innate immune response. *Nature* **406** 782-787.

Alghamdi AS & Foster DN 2005a Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. *Biol Reprod* **73** 1174-1181.

Alghamdi AS, Foster DN, Carlson CS & Troedsson MH 2005b Nitric oxide levels and nitric oxide synthase expression in uterine samples from mares susceptible and resistant to persistent breeding-induced endometritis. *Am J Reprod Immunol* **53** 230-237.

Alghamdi AS, Foster DN & Troedsson MH 2004 Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri. *Reproduction* **127** 593-600.

Amulic B & Hayes G 2011 Neutrophil extracellular traps. *Current biology : CB* **21** R297-298.

Angulo J, Wright HM, Cuevas P, Gonzalez-Corrochano R, Fernandez A, Cuevas B, La Fuente JM, Gupta S & Saenz de Tejada I 2010 Nebivolol Dilates Human Penile Arteries and Reverses Erectile Dysfunction in Diabetic Rats through Enhancement of Nitric Oxide Signaling. *J Sex Med*.

Asbury AC, Gorman NT & Foster GW 1984 Uterine defense mechanisms in the mare: Serum opsonins affecting phagocytosis of Streptococcus zooepidemicus by equine neutrophils. *Theriogenology* **21** 375-385.

Asbury AC, Halliwell REW, Foster GW & Longino SJ 1980 Immunoglobulins in uterine secretions of mares with differing resistance to endometritis. *Theriogenology* **14** 299-308.

Atreya R, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, Schutz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schurmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S & Neurath MF 2000 Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nature medicine* **6** 583-588.

Ball BA, Shin SJ, Patten VH, Lein DH & Woods GL 1988 Use of a low-volume uterine flush for microbiologic and cytologic examination of the mare's endometrium. *Theriogenology* **29** 1269-1283.

Barbacini S, Necchi D, Zavaglia G & Squires EL 2003 Retrospective study on the incidence of postinsemination uterine fluid in mares inseminated with frozen/thawed semen. *Journal of Equine Veterinary Science* **23** 493-496.

Barnes PJ 1998 Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clinical science* **94** 557-572.

Barton BE & Jackson JV 1993 Protective role of interleukin 6 in the lipopolysaccharide-galactosamine septic shock model. *Infection and immunity* **61** 1496-1499.

Berinstein A, Piatti P, Gaggino OP, Schudel AA & Sadir AM 1991 Enhancement of the immune response elicited with foot-and-mouth disease virus vaccines by an extract of the Mycobacterium sp. wall. *Vaccine* **9** 883-888.

Betteridge KJ, Eaglesome MD, Mitchell D, Flood PF & Beriault R 1982 Development of horse embryos up to twenty two days after ovulation: observations on fresh specimens. *Journal of anatomy* **135** 191-209.

Bliss A & Campbell M 2002 A comparison of the ecbolic efficacy of intravenous and intrauterine oxytocin treatments. *Theriogenology* **58** 473-477.

Bohle A 2000 Recent knowledge on BCG's mechanism of action in the treatment of superficial bladder cancer. *Brazilian Journal of urology* **26** 488-502.

Bohle A, Nowc C, Ulmer AJ, Musehold J, Gerdes J, Hofstetter AG & Flad HD 1990 Elevations of cytokines interleukin-1, interleukin-2 and tumor necrosis factor in the urine of patients after intravesical bacillus Calmette-Guerin immunotherapy. *The Journal of urology* **144** 59-64.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y & Zychlinsky A 2004 Neutrophil extracellular traps kill bacteria. *Science* **303** 1532-1535.

Brinsko SP, Rigby SL, Varner DD & Blanchard TL. 2003. A practical method for recognizing mares susceptible to post-breeding endometritis. In *49th Annual Convention of the American Association of Equine Practitioners*.

Brinsko SP, Varner DD & Blanchard TL 1991 The effect of uterine lavage performed four hours post insemination on pregnancy rate in mares. *Theriogenology* **35** 1111-1119.

Bucca S, Carli A, Buckley T, Dolci G & Fogarty U 2008 The use of dexamethasone administered to mares at breeding time in the modulation of persistent mating induced endometritis. *Theriogenology* **70** 1093-1100.

Burleson MD, LeBlanc MM, Riddle WT & Hendricks KEM 2010 Endometrial microbial isolates are associated with different ultrasonographic and endometrial cytology findings in Thoroughbred mares. *Animal Reproduction Science* **121** 103.

Card C 2005 Post-breeding inflammation and endometrial cytology in mares. *Theriogenology* **64** 580-588.

Carnevale EM & Ginther OJ 1992 Relationships of age to uterine function and reproductive efficiency in mares. *Theriogenology* **37** 1101-1115.

Cheung AT, Liu IK, Walsh EM & Miller ME 1985 Phagocytic and killing capacities of uterine-derived polymorphonuclear leukocytes from mares resistant and susceptible to chronic endometritis. *American journal of veterinary research* **46** 1938-1940.

Christoffersen M, Baagoe CD, Jacobsen S, Bojesen AM, Petersen MR & Lehn-Jensen H 2010 Evaluation of the systemic acute phase response and endometrial gene expression of serum amyloid A and pro- and anti-inflammatory cytokines in mares with experimentally induced endometritis. *Vet Immunol Immunopathol* **138** 95-105.

Christoffersen M, Woodward E, Bojesen AM, Jacobsen S, Petersen MR, Troedsson MH & Lehn-Jensen H 2012 Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis. *BMC veterinary research* **8** 41.

Cocchia N, Paciello O, Auletta L, Uccello V, Silvestro L, Mallardo K, Paraggio G & Pasolini MP 2012 Comparison of the cytobrush, cottonswab, and low-volume

uterine flush techniques to evaluate endometrial cytology for diagnosing endometritis in chronically infertile mares. *Theriogenology* **77** 89-98.

Combs GB, LeBlanc MM, Neuwirth L & Tran TQ 1996 Effects of prostaglandin F2α, cloprostenol and fenprostalene on uterine clearance of radiocolloid in the mare. *Theriogenology* **45** 1449-1455.

Cyktor JC & Turner J 2011 Il-10 and Immunity against Prokaryotic and Eukaryotic Intracellular Pathogens. *Infect Immun*.

Dahms BJ & Troedsson MHT 2002 The effect of seminal plasma components on opsonisation and PMN-phagocytosis of equine spermatozoa. *Theriogenology* **58** 457-460.

Dell' Aqua JA, Papa FO, Lopes MD, Alvarenga MA, Macedo LP & Melo CM 2006 Modulation of acute uterine inflammatory response after artificial insemination with equine frozen semen. *Animal Reproduction Science* **94** 270-273.

Diao H & Kohanawa M 2005 Endogenous interleukin-6 plays a crucial protective role in streptococcal toxic shock syndrome via suppression of tumor necrosis factor alpha production. *Infection and immunity* **73** 3745-3748.

Dinarello CA 1991 Interleukin-1 and interleukin-1 antagonism. *Blood* **77** 1627-1652.

Doty A, Buhi WC, Benson S, Scoggin KE, Pozor M, Macpherson M, Mutz M & Troedsson MH 2011 Equine CRISP3 modulates interaction between spermatozoa and polymorphonuclear neutrophils. *Biology of reproduction* **85** 157-164.

Duell BL, Tan CK, Carey AJ, Wu F, Cripps AW & Ulett GC 2012 Recent insights into microbial triggers of interleukin-10 production in the host and the impact on infectious disease pathogenesis. *FEMS immunology and medical microbiology*.

Eleftheriadis T, Liakopoulos V, Antoniadi G, Stefanidis I & Galaktidou G 2010 Arginase type I as a marker of coronary heart disease in hemodialysis patients. *Int Urol Nephrol*.

Elenkov IJ 2004 Glucocorticoids and the Th1/Th2 balance. *Annals of the New York Academy of Sciences* **1024** 138-146.

Esteller-Vico A, Liu I, Brosnan RJ & Steffey EP 2010 Uterine vascular elastosis and its effect on uterine blood flow in cyclic mares. *Animal Reproduction Science* **121** 87-88.

Feghali CA & Wright TM 1997 Cytokines in acute and chronic inflammation. *Frontiers in bioscience : a journal and virtual library* **2** d12-26.

Filion MC & Phillips NC 2001 Therapeutic potential of mycobacterial cell wall-DNA complexes. *Expert opinion on investigational drugs* **10** 2157-2165.

Fumuso E, Aguilar G, Giguere S, David O, Wade J & Rogan D 2006 Interleukin-8 (IL-8) and 10 (IL-10) mRNA transcriptions in the endometrium of normal mares and mares susceptible to persistent post-breeding endometritis. *Animal Reproduction Science 94*282-285.

Fumuso E, G. A, Bruno S, Videla-Dorna I, Wade J, Rogan D & Bowden RA. 2003a. Non-specific immunomodulation at post-partum, improves uterine condition and fertility in mares. In *8th World Equine Veterinary Association*.

Fumuso E, Giguere S, Wade J, Rogan D, Videla-Dorna I & Bowden RA 2003b. Endometrial IL-1beta, IL-6 and TNF-alpha, mRNA expression in mares resistant

or susceptible to post-breeding endometritis. Effects of estrous cycle, artificial insemination and immunomodulation. *Vet Immunol Immunopathol* **96** 31-41.

Fumuso EA, Aguilar J, Giguere S, Rivulgo M, Wade J & Rogan D 2007b. Immune parameters in mares resistant and susceptible to persistent post-breeding endometritis: effects of immunomodulation. *Vet Immunol Immunopathol* **118** 30-39.

Gabler C, Drillich M, Fischer C, Holder C, Heuwieser W & Einspanier R 2009 Endometrial expression of selected transcripts involved in prostaglandin synthesis in cows with endometritis. *Theriogenology* **71** 993-1004.

Gabler C, Fischer C, Drillich M, Einspanier R & Heuwieser W 2010 Time-dependent mRNA expression of selected pro-inflammatory factors in the endometrium of primiparous cows postpartum. *Reprod Biol Endocrinol* **8** 152.

Gensert JM & Ratan RR 2006 The metabolic coupling of arginine metabolism to nitric oxide generation by astrocytes. *Antioxid Redox Signal* **8** 919-928.

Gerard N, Caillaud M, Martoriati A, Goudet G & Lalmanach AC 2004 The interleukin-1 system and female reproduction. *J Endocrinol* **180** 203-212.

Ginther OJ 1992. Reproductive Biology of the Mare.

Green SJ, Scheller LF, Marletta MA, Seguin MC, Klotz FW, Slayter M, Nelson BJ & Nacy CA 1994 Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. *Immunology letters* **43** 87-94.

Gruninger B, Schoon HA, Schoon D, Menger S & Klug E 1998 Incidence and morphology of endometrial angiopathies in mares in relationship to age and parity. *Journal of comparative pathology* **119** 293-309.

Gupta SP, Patel S, Yadav S, Singh AK, Singh S & Singh MP 2010 Involvement of nitric oxide in maneb- and paraquat-induced Parkinson's disease phenotype in mouse: is there any link with lipid peroxidation? *Neurochem Res* **35** 1206-1213.

Hackett TL, Holloway R, Holgate ST & Warner JA 2008 Dynamics of pro-inflammatory and anti-inflammatory cytokine release during acute inflammation in chronic obstructive pulmonary disease: an ex vivo study. *Respiratory research* **9** 47.

Hakansson A, Albihn A & Magnusson U 1993 The contribution of complement to opsonic activity in the uterine secretions of mares free of endometritis. *Theriogenology* **39** 601-609.

Hamann H, Jude R, Sieme H, Mertens U, Topfer-Petersen E, Distl O & Leeb T 2007 A polymorphism within the equine CRISP3 gene is associated with stallion fertility in Hanoverian warmblood horses. *Animal genetics* **38** 259-264.

Hemberg E, Lundeheim N & Einarsson S 2005b Retrospective study on vulvar conformation in relation to endometrial cytology and fertility in thoroughbred mares. *J Vet Med A Physiol Pathol Clin Med* **52** 474-477.

Hirsbrunner G, Kaufmann C, Keller C, Husler J & Steiner A 2010 Spontaneous myometrial contractility in cows suffering from endometritis-Influence of localisation, smooth muscle layer and cycle phase. An in vitro study. *Anim Reprod Sci* **118** 124-130.

Hirsbrunner G, Reist M, Couto SS, Steiner A, Snyder J, vanLeeuwen E & Liu I 2006 An in vitro study on spontaneous myometrial contractility in the mare during estrus and diestrus. *Theriogenology* **65** 517-527.

Hodge S, Hodge G, Flower R & Han P 1999 Methyl-prednisolone up-regulates monocyte interleukin-10 production in stimulated whole blood. *Scandinavian Journal of Immunology* **49** 548-553.

Hoffmann C, Ellenberger C, Mattos RC, Aupperle H, Dhein S, Stief B & Schoon HA 2009 The equine endometrosis: new insights into the pathogenesis. *Animal Reproduction Science* **111** 261-278.

Hou YC, Janczuk A & Wang PG 1999 Current trends in the development of nitric oxide donors. *Current pharmaceutical design* **5** 417-441.

Hughes J & Loy RG 1969 Investigations on the effect of intrauterine inoculations of streptococcus zooepidemicus in the mare. *Proc Am Assoc Equine Pract* **15** 289-292.

Hurtgen JP 2006 Pathogenesis and treatment of endometritis in the mare: a review. *Theriogenology* **66** 560-566.

Jones SA 2005 Directing transition from innate to acquired immunity: defining a role for IL-6. *Journal of immunology* **175** 3463-3468.

Kannaki TR, Shanmugam M & Verma PC 2011 Toll-like receptors and their role in animal reproduction. *Animal Reproduction Science* **125** 1-12.

Kasimanickam R, Duffield TF, Foster RA, Gartley CJ, Leslie KE, Walton JS & Johnson WH 2005 A comparison of the cytobrush and uterine lavage techniques to evaluate endometrial cytology in clinically normal postpartum dairy cows. *The Canadian veterinary journal. La revue veterinaire canadienne* **46** 255-259.

Katila T 1995 Onset and duration of uterine inflammatory response of mares after insemination with fresh semen. *Biology of Reproduction Monograph* **1** 515-517.

Kenney RM & Doig PA 1986. Equine Endometrial Biopsy. In *Current Therapy in Theriogenology: Diagnosis, Treatment, and Prevention of Reproductive Diseases in Small and Large Animals*, pp. 723-729.

Kishimoto T, Akira S, Narazaki M & Taga T 1995 Interleukin-6 family of cytokines and gp130. *Blood* **86** 1243-1254.

Kotilainen T, Huhtinen M & Katila T 1994 Sperm-induced leukocytosis in the equine uterus. *Theriogenology* **41** 629-636.

Kuby J 1992 *Immunology*. New York: W. H. Freeman and Company.

LeBlanc M, Johnson R, Calderwood Mays M & Valderrama C 1995 Lymphatic clearance of India ink in reproductively normal mares and mares susceptible to endometritis. *Biology of Reproduction Monograph*501-506.

LeBlanc MM 2010 Advances in the diagnosis and treatment of chronic infectious and post-mating-induced endometritis in the mare. *Reprod Domest Anim* **45 Suppl 2** 21-27.

LeBlanc MM, Neuwirth L, Asbury AC, Tran T, Mauragis D & Klapstein E 1994 Scintigraphic measurement of uterine clearance in normal mares and mares with recurrent endometritis. *Equine Vet J* **26** 109-113.

LeBlanc MM, Neuwirth L, Jones L, Cage C & Mauragis D 1998 Differences in uterine position of reproductively normal mares and those with delayed uterine clearance detected by scintigraphy. *Theriogenology* **50** 49-54.

Li D, Liu Y, Li Y, Lv Y, Pei X & Guo D 2010 Significance of nitric oxide concentration in plasma and uterine secretes with puerperal endometritis in dairy cows. *Vet Res Commun* **34** 315-321.

Liu I, Rakestraw PA, Coit C, Harmon F & Snyder J 1997 An in vitro investigation of the mechanism of neuromuscular regulation in myometrial contractility. *Pferdeheilkunde* **13** 557.

Liu IK, Cheung AT, Walsh EM, Miller ME & Lindenberg PM 1985 Comparison of peripheral blood and uterine-derived polymorphonuclear leukocytes from mares resistant and susceptible to chronic endometritis: chemotactic and cell elastimetry analysis. *American journal of veterinary research* **46** 917-920.

Liu IKM & Troedsson MHT 2008 The diagnosis and treatment of endometritis in the mare: Yesterday and today. *Theriogenology* **70** 415-420.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25** 402-408.

Ludwig S, Schoon D, Aupperle H, von Reiswitz A & Schoon HA 2001 Angiopathies in the equine endometrial biopsy - a marker for extrauterine vascular lesions? *Pferdeheilkunde* **17** 608-614.

Macmicking J, Xie QW & Nathan C 1997 Nitric oxide and macrophage function. *Annu Rev Immunol* **15** 323-350.

Madill S, Troedsson MH, Alexander SL, Shand N, Santschi EM & Irvine CH 2000 Simultaneous recording of pituitary oxytocin secretion and myometrial activity in oestrous mares exposed to various breeding stimuli. *J Reprod Fertil Suppl*351-361.

Martoriati A, Duchamp G & Gerard N 2003 In vivo effect of epidermal growth factor, interleukin-1beta, and interleukin-1RA on equine preovulatory follicles. *Biology of reproduction* **68** 1748-1754.

McKinnon AO, Squires EL, Harrison LA, Blach EL & Shideler RK 1988 Ultrasonographic studies on the reproductive tract of mares after parturition: effect of involution and uterine fluid on pregnancy rates in mares with normal and delayed first postpartum ovulatory cycles. *Journal of the American Veterinary Medical Association* **192** 350-353.

McKinnon AO & Voss JL. 1993 Equine Reproduction.

Mitchell G, Liu IK, Perryman LE, Stabenfeldt GH & Hughes JP 1982 Preferential production and secretion of immunoglobulins by the equine endometrium--a mucosal immune system. *Journal of reproduction and fertility. Supplement* **32** 161-168.

Morris L & Eden C. 2008 The use of corticosteroids at the time of mating to prevent post breeding endometritis. In *Australian College of Veterinary Scientist 2008 Annual Conference*, pp. 88-89.

Nash D, Lane E, Herath S & Sheldon IM 2008 Endometrial explant culture for characterizing equine endometritis. *Am J Reprod Immunol* **59** 105-117.

Nash DM, Sheldon IM, Herath S & Lane EA 2010 Markers of the uterine innate immune response of the mare. *Anim Reprod Sci* **119** 31-39.

Nielsen JM 2005 Endometritis in the mare: a diagnostic study comparing cultures from swab and biopsy. *Theriogenology* **64** 510-518.

Novak S, Smith TA, Paradis F, Burwash L, Dyck MK, Foxcroft GR & Dixon WT 2010 Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. *Theriogenology* **74** 956-967.

Oguri N & Tsutsumi Y 1972 Non-surgical recovery of equine eggs, and an attempt at non-surgical egg transfer in horses. *J Reprod Fertil* **31** 187-195.
Ohman T, Klein C, Doty A & Troedsson MHT 2011 The phagocytic function of blood-derived polymorphonuclear neutrophils after administration of dexamethasone for the modulation of post-breeding endometritis in the mare. *Pferdeheilkunde* **27** 267-270.
Parham P 2005 *The Immune System*. New York, NY: Garland Science.
Persichini T, Cantoni O, Suzuki H & Colasanti M 2006 Cross-talk between constitutive and inducible NO synthase: An update. *Antioxidants & Redox Signaling* **8** 949-954.
Ricketts SW & Alonso S 1991 The effect of age and parity on the development of equine chronic endometrial disease. *Equine veterinary journal* **23** 189-192.
Riddle WT, LeBlanc MM & Stromberg AJ 2007 Relationships between uterine culture, cytology and pregnancy rates in a Thoroughbred practice. *Theriogenology* **68** 395-402.
Rigby S, Love C, Carpenter K, Varner D & Blanchard T 1998 Use of prostaglandin E2 to ripen the cervix of the mare prior to induction of parturition. *Theriogenology* **50** 897-904.
Rogan D, Fumuso E, Rodríguez E, Wade J & Sánchez Bruni SF 2007 Use of a Mycobacterial Cell Wall Extract (MCWE) in Susceptible Mares to Clear Experimentally Induced Endometritis With Streptococcus zooepidemicus. *Journal of Equine Veterinary Science* **27** 112-117.
Rosselli M, Keller PJ & Dubey RK 1998 Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Hum Reprod Update* **4** 3-24.
Saxena D, Purohit SB, Kumer GP & Laloraya M 2000 Increased appearance of inducible nitric oxide synthase in the uterus and embryo at implantation. *Nitric Oxide* **4** 384-391.
Schlafer DH 2007 Equine endometrial biopsy: Enhancement of clinical value by more extensive histopathology and application of new diagnostic techniques? *Theriogenology* **68** 413-422.
Schroder K, Hertzog PJ, Ravasi T & Hume DA 2004 Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology* **75** 163-189.
Stolla R & Bollwein H 1997 Color doppler sonography of the uterine artery in subfertile mares. *Pferdeheilkunde* **13** 547.
Takehara Y, Dharmarajan AM, Kaufman G & Wallach EE 1994 Effect of interleukin-1 beta on ovulation in the in vitro perfused rabbit ovary. *Endocrinology* **134** 1788-1793.
Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K & Akira S 1999 Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11** 443-451.
Thomas DD, Liu X, Kantrow SP & Lancaster JR, Jr. 2001 The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O2. *Proceedings of the National Academy of Sciences of the United States of America* **98** 355-360.

Tilg H, Trehu E, Atkins MB, Dinarello CA & Mier JW 1994 Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* **83** 113-118.

Traub-Dargatz JL, Salman MD & Voss JL 1991 Medical problems of adult horses, as ranked by equine practitioners. *Journal of the American Veterinary Medical Association* **198** 1745-1747.

Tripathi P 2007 Nitric oxide and immune response. *Indian J Biochem Biophys* **44** 310-319.

Troedsson M, Crabo, B., Ibrahim, N., Scott, M., Ing, M. 1995 Mating-induced endometritis: mechanisms, clinical importance, and consequences. *Proceedings of the American Association of Equine Practitioners* **41** 11-12.

Troedsson MH 1999 Uterine clearance and resistance to persistent endometritis in the mare. *Theriogenology* **52** 461-471.

Troedsson MH 2006 Breeding-induced endometritis in mares. *Vet Clin North Am Equine Pract* **22** 705-712.

Troedsson MH, deMoraes MJ & Liu IK 1993a Correlations between histologic endometrial lesions in mares and clinical response to intrauterine exposure with Streptococcus zooepidemicus. *Am J Vet Res* **54** 570-572.

Troedsson MH, Desvousges A, Alghamdi AS, Dahms B, Dow CA, Hayna J, Valesco R, Collahan PT, Macpherson ML, Pozor M & Buhi WC 2005 Components in seminal plasma regulating sperm transport and elimination. *Animal Reproduction Science* **89** 171-186.

Troedsson MH, Lee CS, Franklin RD & Crabo BG 2000 The role of seminal plasma in post-breeding uterine inflammation. *Journal of reproduction and fertility. Supplement*341-349.

Troedsson MH & Liu IK 1991 Uterine clearance of non-antigenic markers (51Cr) in response to a bacterial challenge in mares potentially susceptible and resistant to chronic uterine infections. *J Reprod Fertil Suppl* **44** 283-288.

Troedsson MH, Liu IK, Ing M, Pascoe J & Thurmond M 1993b Multiple site electromyography recordings of uterine activity following an intrauterine bacterial challenge in mares susceptible and resistant to chronic uterine infection. *J Reprod Fertil* **99** 307-313.

Troedsson MH, Liu IK & Thurmond M 1993c Function of uterine and blood-derived polymorphonuclear neutrophils in mares susceptible and resistant to chronic uterine infection: phagocytosis and chemotaxis. *Biology of reproduction* **49** 507-514.

Troedsson MH, Liu IK & Thurmond M 1993d Immunoglobulin (IgG and IgA) and complement (C3) concentrations in uterine secretion following an intrauterine challenge of Streptococcus zooepidemicus in mares susceptible to versus resistant to chronic uterine infection. *Biology of reproduction* **49** 502-506.

Troedsson MH, Loset K, Alghamdi AM, Dahms B & Crabo BG 2001 Interaction between equine semen and the endometrium: the inflammatory response to semen. *Anim Reprod Sci* **68** 273-278.

Troedsson MH, Scott MA & Liu IK 1995b Comparative treatment of mares susceptible to chronic uterine infection. *Am J Vet Res* **56** 468-472.

Troedsson MHT 1997 Therapeutic considerations for mating-induced endometritis. *Pferdeheilkunde* **13** 516-520.

Tuominen VJ, Ruotoistenmaki S, Viitanen A, Jumppanen M & Isola J 2010 ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67. *Breast cancer research : BCR* **12** R56.

Van der Hoek KH, Woodhouse CM, Brannstrom M & Norman RJ 1998 Effects of interleukin (IL)-6 on luteinizing hormone- and IL-1beta-induced ovulation and steroidogenesis in the rat ovary. *Biology of reproduction* **58** 1266-1271.

Waelchli RO 1990 Endometrial biopsy in mares under nonuniform breeding management conditions: Prognostic value and relationship with age. *The Canadian veterinary journal. La revue veterinaire canadienne* **31** 379-384.

Watson ED 2000 Post-breeding endometritis in the mare. *Animal Reproduction Science* **60-61** 221-232.

Watson ED, Stokes CR & Bourne FJ 1987 Cellular and humoral defence mechanisms in mares susceptible and resistant to persistent endometritis. *Veterinary immunology and immunopathology* **16** 107-121.

Williamson P, Dunning A, O'Connor J & Penhale WJ 1983 Immunoglobulin levels, protein concentrations and alkaline phosphatase activity in uterine flushings from mares with endometritis. *Theriogenology* **19** 441-448.

Woodward E, Christofferesn M, Campos J, Bentancourt A, Horohov D, Scoggin KE, Squires E & Troedsson MHT. 2011a. Endometrial cytokine expression in mares with different resistance to persistent breeding induced endometritis (PBIE) at multiple time points after insemination. In *44th Annual Meeting for the Society for the Study of Reproduction*.

Woodward E, Christofferesn M, Campos J, Squires E & Troedsson MHT. 2011b. Susceptibility to delayed uterine clearance after breeding: Relationship to endometrial biopsy score and age, and variations between seasons. In *Annual Meeting of the Society for Theriogenology*.

Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF & Achong MK 1998 IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *The Journal of clinical investigation* **101** 311-320.

Yuksel ZS, Buber E, Kocagoz T, Alp A, Saribas Z & Acan NL 2011 Mycobacterial strains that stimulate the immune system most efficiently as candidates for the treatment of bladder cancer. *Journal of molecular microbiology and biotechnology* **20** 24-28.

Zanetti M, Cappellari GG, Burekovic I, Barazzoni R, Stebel M & Guarnieri G 2010 Caloric restriction improves endothelial dysfunction during vascular aging: Effects on nitric oxide synthase isoforms and oxidative stress in rat aorta. *Exp Gerontol*.

Zent W, Troedsson M & Xue JL 1998 Postbreeding uterine fluid accumulation in a normal population of Thoroughbred mares: a field study. *Proceedings of the Annual Convention of the AAEP* **44** 64-65.

VITA

Elizabeth Moran Woodward
Born January 6, 1982
From: Chatham, Pennsylvania

EDUCATION

University of Kentucky 2007-present
Lexington, KY
College of Agriculture
Department of Veterinary Science

Delaware Valley College 2005
Doylestown, Pennsylvania
Bachelor of Science
Concentration in Equine Science

PUBLICATIONS

Abstracts

Woodward, E.M., Christoffersen, M., Horohov, D., Squires, E.L., Troedsson, M.H.T. *The effect of immune modulators on endometrial cytokine expression in mares susceptible to persistent breeding induced endometritis.* Accepted abstract for the 17th International Congress on Animal Reproduction. To be held in Vancouver, Canada, July 2012.

Woodward, E.M., Christoffersen, M., Campos, J., Betancourt, A., Horohov, D., Scoggin, K., Squires, E.L., Troedsson, M.H.T. *Endometrial cytokine expression in mares with different resistance to persistent breeding induced endometritis (PBIE) at multiple time points after insemination.* Proceedings from the 44th annual meeting for the Society for the Study of Reproduction "Reproduction and the World's Future". Platform presentation #77 Portland, OR August 2011

Woodward, E., Christoffersen, M., Campos, J., Squires, E.L., Troedsson, M.H.T. *Susceptibility to delayed uterine clearance after breeding: Relationship to endometrial biopsy score and age, and variations between seasons.* Proceedings from the meeting of Society for Theriogenology. Milwaukee WI, 2011.

Christoffersen, M., **Woodward, E**., Jacobsen, S., Bojesen, A.M., Petersen, M.R., Lehn-Jensen, H., Troedsson, M.H.T. *Endometrial gene expression of inflammatory cytokines and serum amyloid A in mares resistant or susceptible to persistent endometritis.* Proceedings from the meeting of Society for Theriogenology. Milwaukee WI, 2011.

Miller, L.M.J., **Woodward, E.**, Campos, J.R., Troedsson, M.H.T. *Expression of sperm protein at 22kDa (SP22) on equine spermatozoa prior to and following heat-induced testicular degeneration*. Animal Reproduction Science 121S (2010) S148. Proceedings for the Tenth International Symposium on Equine Reproduction. Lexington, KY July 2010

Sessions, D.R., **Woodward, E.M**., Fitzgerald, B.P. *Relationship between elevated insulin and matrix metalloproteinases-2 and -9 and their inhibitors in equine granulosa cells in vivo.* Animal Reproduction Science 121S (2010) S40. Proceedings for the Tenth International Symposium on Equine Reproduction. Lexington, KY July 2010

Woodward, E., LeBlanc, M., Horohov, D., Fitzgerald, B.P. *Can peripheral blood cytokine expression indicate mating-induced endometritis?* Proceedings from The Chronically Infertile Mare. Havemeyer Foundation meeting November 2008 Hilton Head South Carolina. Pg. 28

Peer Reviewed Publications

Woodward, E.M., Christoffersen, M., Campos, J., Betancourt, A., Horohov, D., Scoggin, K.E., Squires, E., Troedsson, M.M.T. *Endometrial inflammatory markers of the early innate immune response in mares susceptible and resistant to persistent breeding induced endometritis (PBIE).* Submitted, Reproduction.

Woodward, E., Christoffersen, M., Campos, J., Squires, E.L., Troedsson, M.H.T. *Susceptibility to persistent breeding induced endometritis: relationship to endometrial biopsy score and age, and variations between seasons.* Theriogenology. Available online April, 2012.

Troedsson, M.H.T., **Woodward, E.** *Breeding-induced endometritis.* Pferdeheilkunde, 7th International Conference on Equine Reproductive Medicine. 2012.

Christoffersen, M., **Woodward, E**., Bojesen, A.M., Jacobsen, S., Petersen, M.R., Troedsson, M., Lehn-Jensen, H. *Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis.* BMC Veterinary Research. 2012, 8:41.

Christoffersen, M., **Woodward, E.M.**, Bojesen, A.M., Petersen, M.R., Squires E.L., Lehn-Jensen, H., Troedsson, M.H. *Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares.* Accepted, Theriogenology.

INVITED PRESENTATIONS

"Endometrial cytokine expression in mares with different resistance to persistent breeding induced endometritis (PBIE) at multiple time points after insemination". 44th annual meeting of the Society for the Study of Reproduction: Reproduction and the World's Future. Platform presentation #77 Portland, OR August 1, 2011

"Susceptibility to delayed uterine clearance after breeding: Relationship to endometrial biopsy score and age, and variations between seasons". Annual meeting of the Society for Theriogenology. Milwaukee WI, 2011. August 9, 2011

"The role of nitric oxide and cytokines in persistent breeding-induced endometritis". Gluck Equine Research Center University of Kentucky Departmental Seminar. Lexington, KY April 8, 2011.

"Can peripheral blood cytokine expression indicate mating-induced endometritis?" Havemeyer Foundation meeting: The Chronically Infertile Mare. Hilton Head, SC November 6, 2008

PROFESSIONAL SOCIETIES

Society for the Study of Reproduction Trainee member	Since 2011
Society for Theriogenology Graduate Student member	Since 2011